



Human neutrophil flavocytochrome b : structure and function
by Thomas Richard Foubert

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of
Philosophy in Microbiology
Montana State University
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Abstract:

The catalytic core of the multi-subunit NADPH oxidase of neutrophils and other phagocytes is a hemoprotein known as flavocytochrome b₅₅₈. The structure of this protein has not been determined, thus preventing exploration of structure-function relationships that would provide a clearer picture of the molecular mechanisms that control oxidase function *in vivo*. The work presented herein summarizes the information that we obtained regarding 1) localization of the heme groups within the protein structure, and 2) identification of a possible allosteric mechanism of NADPH oxidase activation that incorporates changes in the molecular geometry of flavocytochrome b.

Detergent-solubilized flavocytochrome b was exposed to Staphylococcal V₈ protease for 1 hour. The digestion milieu was then fractionated by HPLC size-exclusion chromatography, resulting in isolation of a core fragment with a native heme absorbance spectrum, comprising two polypeptides of 60-66 and 17 kDa by SDS-PAGE. Sequence and immunoblot analyses of the deglycosylated polypeptide identified the polypeptide as the NH₂-terminal 320 to 363 amino acid residues of gp91phox, and the NH₂-terminal 169 to 171 amino acid residues of p22phox, providing the first direct evidence that the membrane-spanning regions of flavocytochrome b are responsible for heme ligation.

The anionic amphiphiles sodium- or lithium-dodecyl sulfate, and arachidonate (SDS, LDS, AA) initiate NADPH oxidase and proton channel activity in cell-free systems and intact neutrophils. To investigate whether these amphiphiles exert allosteric effects on flavocytochrome b, we developed a system using trisulfopyrenyl (Cascade Blue®, CCB)-labeled wheat germ agglutinin, or CCB-labeled monoclonal antibodies as extrinsic fluorescence probes for resonance energy transfer to the intrinsic hemes of detergent-solubilized and lipid-bound flavocytochrome b. In solution, flavocytochrome b complexed with both CCB conjugates and partially quenched the CCB fluorescence. Subsequent additions of SDS, LDS, or AA, but not the oxidase antagonist methyl ester of AA, to concentrations typical of cell-free oxidase assays caused a saturable, concentration-dependent relaxation of the fluorescence quenching. The relaxation effects were independent of complex dissociation, or alterations in the spectral properties of the chromophores, suggesting induction of a flavocytochrome b conformational change in which the proximity or orientation of the hemes have undergone significant movement relative to an extracellular reference point.

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

January 2002

D378
F8215

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This dissertation has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The catalytic core of the multi-subunit NADPH oxidase of neutrophils and other phagocytes is a hemoprotein known as flavocytochrome *b*₅₅₈. The structure of this protein has not been determined, thus preventing exploration of structure-function relationships that would provide a clearer picture of the molecular mechanisms that control oxidase function *in vivo*. The work presented herein summarizes the information that we obtained regarding 1) localization of the heme groups within the protein structure, and 2) identification of a possible allosteric mechanism of NADPH oxidase activation that incorporates changes in the molecular geometry of flavocytochrome *b*.

Detergent-solubilized flavocytochrome *b* was exposed to *Staphylococcal* V8 protease for 1 hour. The digestion milieu was then fractionated by HPLC size-exclusion chromatography, resulting in isolation of a core fragment with a native heme absorbance spectrum, comprising two polypeptides of 60-66 and 17 kDa by SDS-PAGE. Sequence and immunoblot analyses of the deglycosylated polypeptide identified the polypeptide as the NH₂-terminal 320 to 363 amino acid residues of gp91^{phox}, and the NH₂-terminal 169 to 171 amino acid residues of p22^{phox}, providing the first direct evidence that the membrane-spanning regions of flavocytochrome *b* are responsible for heme ligation.

The anionic amphiphiles sodium- or lithium-dodecyl sulfate, and arachidonate (SDS, LDS, AA) initiate NADPH oxidase and proton channel activity in cell-free systems and intact neutrophils. To investigate whether these amphiphiles exert allosteric effects on flavocytochrome *b*, we developed a system using trisulfopyrenyl (Cascade Blue®, CCB)-labeled wheat germ agglutinin, or CCB-labeled monoclonal antibodies as extrinsic fluorescence probes for resonance energy transfer to the intrinsic hemes of detergent-solubilized and lipid-bound flavocytochrome *b*. In solution, flavocytochrome *b* complexed with both CCB conjugates and partially quenched the CCB fluorescence. Subsequent additions of SDS, LDS, or AA, but not the oxidase antagonist methyl ester of AA, to concentrations typical of cell-free oxidase assays caused a saturable, concentration-dependent relaxation of the fluorescence quenching. The relaxation effects were independent of complex dissociation, or alterations in the spectral properties of the chromophores, suggesting induction of a flavocytochrome *b* conformational change in which the proximity or orientation of the hemes have undergone significant movement relative to an extracellular reference point.

CHAPTER 1

INTRODUCTION

The NADPH Oxidase of Human Phagocytes

Neutrophils and other phagocytes of the host immune system play an important protective role by seeking out and destroying invading pathogenic organisms (1-4). Their ability to fulfill this task depends in large part on the function of a multi-subunit enzyme complex named the nicotinamide-adenine dinucleotide (NADPH) oxidase. Encounters between the phagocytes and any of several stimuli deriving from bacteria or damaged host cells results in activation of the oxidase. Once activated, the enzyme catalyzes the one-electron reduction of molecular oxygen to the highly energetic radical species superoxide anion (O_2^-) at the expense of intracellular NADPH. Subsequent complex chemical interactions between O_2^- and other constituents of the surrounding milieu produce a myriad of toxic oxygen species that are exploited in conjunction with other neutrophil cytoplasmic granule contents to destroy invading microorganisms (5-11).

Oxidase activation at the cellular level is usually receptor-mediated, involving several intracellular signaling pathways that culminate in assembly of the oxidase subunits at the neutrophil plasma membrane. *In vivo*, the oxidase comprises the cytosolic proteins, p40^{phox}, p47^{phox}, p67^{phox}, the membrane-associated Rac1 (or 2), or Rap1A (12-14), and a single transmembrane component, flavocytochrome *b*₅₅₈ (6-9,15,16). Despite considerable investigation, the sequence of events that precede assembly of the oxidase

complex and the involvement of the individual subunits in controlling oxidase activity remain unclear. One consensus is that the presence of a minimum ensemble consisting of flavocytochrome *b*, $p47^{phox}$, and $p67^{phox}$, is absolutely necessary for normal oxidase activity *in vivo*. Deficiencies in any one of these proteins, arising from genetic lesions that either abolish expression or produce nonfunctional proteins, renders the oxidase catalytically inactive resulting in a clinical syndrome known as chronic granulomatous disease (CGD) (17-28). CGD is considered to be a rare disorder, affecting approximately one in 250,000-500,000 individuals, with no indication of racial preference. While the phagocytes of these individuals appear to otherwise function normally, certain organisms cannot be killed following phagocytosis. As a result, the phagocytes and their ingested pathogens accumulate, forming granulomatous lesions where the organisms thrive and are refractory to conventional treatment regimes. These granulomata, the histological hallmark of the disease, are found in lymph nodes, lungs, liver, bones and the subcutaneous tissues of the patients. Clinical symptoms may also present in the gastrointestinal tract as diarrhea, parianal abscesses, and obstructions due to granuloma formation. CGD is usually diagnosed in children under 2 years of age but has in some cases been recognized later due to the heterogeneous nature of the disease. The heterogeneity in clinical phenotypes is attributed to the immense complexity of the genetic lesions (for written reviews see (28,29), or see the X-CGD database at <http://www.uta.fi/imt/bioinfo/CYBBbase/>), and also the compensatory efficacy of redundant antimicrobial systems that can vary between individuals. Many of the

individuals suffering from this condition eventually succumb to the onslaught of infections and die within the first few years of life (21,24).

Although phagocytes perform a necessary function in host protection, misdirected or inappropriate generation of oxygen-derived radical species from these cells in otherwise healthy individuals contribute to a large number of disease processes such as atherosclerosis, Parkinson's disease, asthma, amyotrophic lateral sclerosis (ALS), adult respiratory distress syndrome (ARDS), and even aging (30-39). Phagocytes that emigrate to sites of injury play a significant part in mediating the initial phases of the inflammatory response by releasing a large number of compounds into the local environment, some of which are responsible for recruitment of other phagocytes and others that possess cytotoxic properties. The accumulative nature of this response sometimes results in non-specific damage to local tissues to an extent exceeding the initial insult, thus aggravating the injury (40-43)

Reactive oxygen metabolites appear to function as signaling molecules that are necessary for tissue neogenesis, fetal development, and apoptosis, hence implicating misdirected superoxide and other radical species in tumorigenesis (35,44-49). There is an emerging body of evidence that suggests that the oxidase also functions in the capacity of both an oxygen sensor (50-58) and a proton channel (49,59-72), though the latter function has recently been challenged (73). Additionally, homo- or orthologues of the oxidase subunits, primarily gp91^{phox}, continue to be identified in cells other than phagocytes (48,49,57,74-86). The prevalence of these homologues or the holoenzyme in such a variety of tissues suggests a multi-faceted biological role for these proteins.

Therefore, understanding the underlying molecular mechanisms that regulate oxidase or oxidase-related activity is of great importance, both for understanding cellular function, and for development of therapeutic strategies for control of inflammatory disease processes.

Human Neutrophil Flavocytochrome *b*

The central component of the NADPH oxidase is a transmembrane *b*-type cytochrome, flavocytochrome *b*₅₅₈ (a.k.a. neutrophil cytochrome *b*, cytochrome *b*₅₅₉, flavocytochrome *b*₂₄₅, and flavocytochrome *b*). The subscript 558 or 559 refers to the reduced α -band absorbance maximum (in nm) and the subscript 245 is an unconventional designation referring to the initially measured midpoint reduction potential (in mV) (87). The protein is a heterodimer (88) with a resting state 1:1 subunit stoichiometry (89-91) comprising a small non-glycosylated subunit, p22^{phox}, and an extensively glycosylated large subunit, gp91^{phox} (the superscript *phox* is an abbreviation of *phagocyte oxidase*, p and gp refer to protein or glycosylated protein, respectively). The primary sequence of both subunits have been deduced from cDNA, but there is no high-resolution structural information currently available for flavocytochrome *b*. Modeling studies suggest that the NH₂-terminal, roughly 60% of the protein contains several regions (two in p22^{phox} and up to six in gp91^{phox}) that are likely to form membrane-spanning α -helical structures (17,18,20,92-95) (D. Baniulis, *et al.*, unpublished). The remaining C-terminal portion of the protein is relatively hydrophilic,

and predicted to reside on the cytosolic aspect of the neutrophil plasma membrane serving as a scaffold for assembly of the oxidase complex (10,96,97). Flavocytochrome *b* is the only membrane-spanning subunit of the oxidase, and therefore functions as the transmembrane conduit for electrons that are used to reduce extracellular molecular oxygen to O_2^- (6,97-101). Flavocytochrome *b* coordinates two (Taylor, *et al.*, unpublished), non-covalently bound heme moieties that are necessary elements of the electron transfer (94,102-105), and possibly proton conduction (106) pathways. Both FAD (107-121) and NADPH (113,118,122,123) are necessary for oxidase activity. Oxidase activity has been achieved *in vitro* in the absence of the cytosolic oxidase proteins by separate groups (124-128), and flavocytochrome *b* contains the necessary determinants of a complete electron transfer chain, i.e. $NADPH \rightarrow FAD \rightarrow \text{heme} \rightarrow O_2$. Direct interactions that occur between flavocytochrome *b* and the other oxidase subunits, or between the oxidase subunits alone, have been revealed through the use of a variety of techniques (124,129-139). A consensus paradigm has emerged where flavocytochrome *b* is considered to be the only catalytic component of the oxidase, and oxidase activity is controlled by concerted interactions between flavocytochrome *b* and the other subunits. Investigation of these interactions has led to identification of the specific residues that define the protein-protein contact regions. These regions are therefore restricted to the intracellular aspect of the plasma membrane, and are also exposed to the cytoplasm, some only in the activated complex. Other studies have utilized antibodies, or antibodies in combination with other techniques, to determine both structural and functional aspects of the oxidase. Collectively, this information has contributed significantly to describing the

surface topology of some of the regions of the cytochrome. Although a substantial amount of information has been gathered, it remains unclear how these interactions confer oxidase activity *in vivo* or *in vitro*. The following sections summarize most of the currently available information that in some way contributes to defining the structure flavocytochrome *b*. The information is delineated by subunit, beginning at the NH₂-terminal regions of each of the subunits, with the initiation methionines designated as number 1. Although the initiation methionines do not appear to be present in the mature protein (88,140), they were included for consistency with the encoded sequences listed in the various protein data bases (e.g. NCBI accession numbers NM000101.1 and P04839 for p22^{phox} and gp91^{phox}, respectively).

Structural and Functional Regions of gp91^{phox}.

Human gp91^{phox} is encoded as a 570 amino acid residue protein with a predicted mass of 65.3 kDa based on primary sequence (20,95). The protein resolves by SDS-PAGE as a diffuse band of ~ 65 to 120 kDa (88) depending on the concentration of polyacrylamide used, and the cell type from which the protein was derived (141). The diffuse appearance is attributed to the extensively heterogeneous N-linked (88,142) glycosylation present at probably three of the five potential NXS/T sites, Asn¹³¹, Asn¹⁴⁸, and Asn²³⁹ (93), thus restricting their location to the extracellular aspect of flavocytochrome *b*.

A number of studies suggest that specific regions of gp91^{phox} may bind one or more of the cytosolic subunits. Several studies have been conducted where the oxidase-inhibitory effects of peptides or antibodies were examined. Their effects were attributed to prevention of oxidase assembly based on the observation that they inhibit activation but not activity. While this perspective may be correct, an accurate definition of what is the "complex" in the active oxidase is still not fully clear at the present time and at least one study has suggested that some of the cytosolic subunits may interact with flavocytochrome *b* in a non-stoichiometric manner (143).

There is some experimental evidence suggesting that direct interactions occur between p67^{phox} and flavocytochrome *b* during oxidase activation. Paclet *et al.* inferred p67^{phox}-induced changes in flavocytochrome *b* structure on the basis of changes in liposome size during oxidase activation using atomic force microscopy (136). Two groups have been able to demonstrate *in vitro* oxidase activity in the presence of high concentrations of p67^{phox} and Rac, or Rac1 in the isoprenylated form, in the absence of p47^{phox} or anionic amphiphiles, suggesting p67^{phox} and possibly Rac1 interact directly with flavocytochrome *b* to regulate oxidase activity (139,144). Cross *et al.* conducted a kinetic study of activation *in vitro* and hypothesized that p67^{phox}, and possibly Rac2 interact in a catalytic fashion directly with flavocytochrome *b* to induce an active state geometry of the cytochrome (143).

Other investigations have suggested that gp91^{phox} binds to p67^{phox}, but the specific residues of either protein that are responsible for this putative interaction are still under investigation. Binding studies conducted by Dang *et al.* (145) showed that

phosphorylated p67^{phox} bound denatured gp91^{phox} on immunoblot. Dot blots of flavocytochrome *b* were also positive when probed with non-phosphorylated p67^{phox}. A GST-p67^{phox} fusion protein was also able to coprecipitate detergent-solubilized flavocytochrome *b*, and the interaction was significantly enhanced by the presence of either Rac1-GTPγS or GDPβS. In a separate study (146), a recombinant, bacterially-expressed C-terminal region of gp91^{phox} consisting of residues 307-570 showed weak superoxide dismutase (SOD) insensitive, NADPH-dependent nitroblue tetrazolium (NBT) reductase activity that was inhibitable by the oxidase inhibitor, diphenylene iodonium (DPI). The NADPH K_m for the truncated gp91^{phox} was similar to that of the native protein, and oxidase activity saturated at a 1:1 gp91^{phox}:FAD molar stoichiometry. This limited activity was increased slightly by addition of rac-1 and p67^{phox}, and doubled when truncated forms of p67^{phox} that retained the activation domain (147) were added. From these observations, the authors concluded that the interaction domain between flavocytochrome *b* and p67^{phox} was intact within this segment.

Oxidase inhibition studies conducted by Park *et al.* (148) utilized a series of peptides mimicking the predicted cytosolic domains of gp91^{phox}. Cytosolic subunit translocation assays with twelve different peptides showed that translocation of both p47^{phox} and p67^{phox} were affected with a corresponding reduction in oxidase activation. In particular, peptide 2, mimicking gp91^{phox} residues 87-100, was able to inhibit p67^{phox} translocation more extensively than p47^{phox} suggesting that the region might directly coordinate p67^{phox} – flavocytochrome *b* interactions.

In contrast to p67^{phox}-gp91^{phox} interactions, considerable effort has been focused on determining the possible contact regions between p47^{phox} and gp91^{phox}. Sequence analysis of an X91⁺ CGD patient revealed a missense mutation predicting a Asp⁵⁰⁰→Gly substitution in gp91^{phox} (137). Neutrophils isolated from the patient exhibited severely attenuated translocation of both p47^{phox} and p67^{phox} to the membranes following PMA stimulation, and these same deficiencies were observed when the neutrophil membranes were tested in a cell-free assay. A synthetic peptide mimicking residues Phe⁴⁹¹ to Gly⁵⁰⁴ of gp91^{phox} was also able to inhibit oxidase activation in a cell-free system from normal neutrophils at an IC₅₀ concentration of ~ 10 μM. Cell-free translocation assays indicated that both p47^{phox} and p67^{phox} did not associate with the membrane fraction, suggesting that the region was critical for cytosolic subunit binding.

Park *et al.* conducted oxidase inhibition and cytosolic subunit translocation assays using twelve different peptides corresponding to the predicted hydrophilic intracellular domains of gp91^{phox} (148). Six of the twelve peptides designated 1, 2, 6, 7, 9, and 12 (corresponding to gp91^{phox} residues 27-46, 87-100, 282-296, 304-321, 434-455, and 559-565 respectively) were shown to inhibit oxidase activation but not activity. Within this group, four of the peptides, 1, 2, 9, and 12 also partially inhibited translocation of the cytosolic subunits in a cell-free assay. Peptides 1 and 9 inhibited p47^{phox} translocation to a greater extent than p67^{phox} suggesting a possible direct interaction between p47^{phox} and gp91^{phox}. The activation of the oxidase corresponded well with the extent of cytosolic subunit translocation with one exception, peptide 1. Interestingly, although peptide 1 (residues 27-46) only partially inhibited translocation of

the cytosolic subunits, it was highly effective in inhibiting oxidase activation ($IC_{50} = 34 \mu M$). A continuation of this study by the same group (149) revealed that truncated versions of peptide 9, corresponding to residues 418-435, and 441-450 of gp91^{phox} (designated L418 and L441, respectively), also exerted inhibitory effects. Peptide L418 was able to inhibit oxidase activation *and* activity *in vitro* in the presence *or* absence of cytosol at an IC_{50} concentration of $10 \mu M$. By testing scrambled and truncated versions of L418 in an *in vitro* superoxide assay, a minimum essential sequence was found, corresponding to residues ⁴²¹KSVWYK⁴²⁶ of gp91^{phox} (designated L420). These tests further revealed that the terminal lysines as well as the tryptophan and tyrosine residues were critical for activity. Similar to L418, L420 was also shown to inhibit both oxidase activation and activity at an IC_{50} concentration of $35-40 \mu M$. A kinetic study of *in vitro* oxidase inhibition indicated that the inhibitory effects of either peptide were uncompetitive with respect to NADPH binding, and with consistently lower overall K_M values in the presence of either peptide. These observations were interpreted to imply that L418 and L420 interacted directly with the flavocytochrome *b* and caused a structural perturbation that increased the affinity for NADPH. In contrast, peptide L441 exhibited irreversible inhibition at an IC_{50} concentration of $\sim 58 \mu M$ suggesting a different inhibitory mechanism. The L418 and L420 peptides are the only ones reported to date that are able to inhibit both activation and activity of the oxidase.

A synthetic peptide corresponding to the C-terminal 551-570 residues of gp91^{phox} (designated L_C) was shown to inhibit oxidase activation in a cell-free system from human neutrophils at an IC_{50} concentration of $4 \mu M$ in the presence of SDS (150). The peptide

was also shown to bind specifically to two proteins of 17 and 47 kDa by immunoblot using α -L_C antibodies, (probably p47^{phox}), but only after the cytosolic fractions had been incubated with SDS. Although this work was done prior to the designation of the 47 kDa protein as p47^{phox}, their results suggest a direct interaction between a 47 kDa protein and the extreme C-terminus of gp91^{phox}. This region of gp91^{phox}, as well as two others were also identified by DeLeo *et al.* in our laboratory by screening p47^{phox} with a random-sequence peptide phage display library (129). Consensus sequences of phage peptides that bound p47^{phox} identified three potential sites of interaction that corresponded to the regions, ⁸⁷STRVRRQL⁹⁴, ⁴⁵¹FEWFADLL⁴⁵⁸, and ⁵⁵⁵ESGPRGVHFIF⁵⁶⁵ of gp91^{phox}. Synthetic peptides corresponding to the ⁷⁸Phe-Leu⁹⁴ and ⁴⁵²Glu-Gln⁴⁶⁴ regions of gp91^{phox} were also shown to inhibit oxidase activation *in vitro* at IC₅₀ concentrations of 1 and 230 μ M, respectively. To determine the regions of the ⁷⁸Phe-Leu⁹⁴ that conferred the inhibitory effects, additional *in vitro* oxidation inhibition studies were conducted using peptides corresponding to gp91^{phox} residues ⁷⁸Phe-Cys⁸⁶ and ⁸⁷Cys-Leu⁹⁴. While the NH₂-terminal half of the ⁷⁸Phe-Leu⁹⁴ peptide (⁷⁸Phe-Cys⁸⁶) exhibited no inhibitory effects at concentrations up to 500 μ M, the C-terminal portion of the ⁷⁸Phe-Leu⁹⁴ (Cys⁸⁷-Leu⁹⁴) showed a similar efficacy to the full-length peptide (IC₅₀ $\sim$ 3 μ M) indicating that the effector region was contained within the residues ⁸⁷CSTRVRRQL⁹⁴. These results suggested a direct interaction between p47^{phox} and the residues ⁸⁷CSTRVRRQL⁹⁴ of gp91^{phox}.

In a separate study, mutant forms of gp91^{phox} were generated by site-directed mutagenesis of human myeloid leukemia PLB-985 cells (151) targeting a similar domain of gp91^{phox} encompassing residues ⁸⁷CSTRVRRQLDRNLTFHK¹⁰². The most profound effects on oxidase function were observed in the charge reversal Arg⁹¹→Glu and Arg⁹²→Glu substitutions where membrane-translocation of the cytosolic oxidase proteins p47^{phox}, p67^{phox}, Rac1, and Rac2 were abolished. These observations suggested that p47^{phox} association with this region of gp91^{phox} and subsequent assembly of the oxidase were mediated by the residues Arg⁹¹ and Arg⁹². This interaction site would necessarily be restricted to the intracellular aspect of the neutrophil membrane, and a separate modeling study further suggested a location near the intracytoplasmic face of the membrane (93).

Rotrosen *et al.* (152) was able to inhibit cell-free oxidase activation using rabbit polyclonal antibodies produced from a synthetic peptide CSNPRGVHFIFNKENF, that corresponded in part (underlined) to the extreme C-terminal ⁵⁵⁸Pro-Phe⁵⁷⁰ residues of gp91^{phox}. The antibodies were shown to be positive for gp91^{phox} but only on the intracellular aspect of sheared neutrophil membrane patches indicating an intracellular location for the region. Six peptides mimicking gp91^{phox} residues 552-570, 559-570, 559-565, 552-558, 119-126 and 2-9 were tested for their ability to inhibit oxidase activity in both the broken cell superoxide assay, and electroporabilized neutrophils. Only peptides 552-570, 559-570, and 559-565 had an inhibitory effect on oxidase activation, and none of the peptides affected oxidase activity after stimulation with arachidonate. Moreover, when these same peptides were allowed to diffuse into electroporated

neutrophils, they were able to inhibit both PMA- and fMLF-stimulated oxidase activation. These assays suggested that the inhibitory activity of the peptides was conferred by a minimum essential peptide sequence consisting of residues mimicking the $^{559}\text{RGVHFIF}^{565}$ residues of $\text{gp91}^{\text{phox}}$. The three peptides containing the $^{559}\text{RGVHFIF}^{565}$ sequence were also able to completely inhibit p47^{phox} phosphorylation in the cell-free assay suggesting that this region of flavocytochrome *b* may somehow modulate this activity. A separate kinetic study of oxidase activation in a cell-free system (153) derived from neutrophils of X91^+ CGD patients lacking either p47^{phox} or p67^{phox} , revealed that the inhibitory effects of the $^{559}\text{RGVHFIF}^{565}$ peptide were exerted only when p47^{phox} -deficient cytosol was preincubated with AA and membranes that was later supplemented with exogenous p47^{phox} . The authors proposed that the peptide inhibited formation of a putative AA-dependent metastable activation intermediate that involved association of p47^{phox} with the membranes, possibly coordinated by the $^{559}\text{RGVHFIF}^{565}$ region of $\text{gp91}^{\text{phox}}$. Further studies suggested that the inhibitory effects of the peptide were conferred by the side chains of the Arg^{559} , Val^{561} , Ile^{564} , and both Phe^{563} and Phe^{565} residues (154).

The function of this region of $\text{gp91}^{\text{phox}}$ was also explored *in situ* (155) with site-directed mutagenesis of $\text{gp91}^{\text{phox}}$ expressed in an X-CGD PLB-985 cell line (156), targeting $^{559}\text{RGVHFIF}^{565}$ as well as adjacent residues. Mutant cells carrying single Ala substitutions at residues Arg^{559} , His^{562} , Phe^{563} , or Ile^{564} , and a double Ala substitution at Arg^{559} and Val^{561} had little effect on the maximal oxidase activity when compared wild-type PLB-985 cells. However, single point substitutions at Val^{561} with Ala, Thr, or Glu

resulted in a 2-4 fold reduction in superoxide generation, with the most dramatic effect due to the Val⁵⁶¹→Glu substitution. A single Ile⁵⁶⁴→Ala substitution resulted in a 40% reduction in superoxide activity, and when mutated in tandem with a Phe⁵⁶⁵→Val, all activity was lost. Single substitutions of Phe⁵⁶⁵ with either Ala or Asp caused a ~ 70 and 95% loss of activity, respectively, and a single Phe⁵⁷⁰→Ala substitution caused a ~ 50% loss. These results were somewhat confounded by variable expression levels of flavocytochrome *b* as evidenced by immunoblot comparisons between gp91^{phox} levels in cells expressing mutant gp91^{phox} and wild-type PLB-985 cells. These variations in gp91^{phox} expression levels were not taken into account for their functional comparisons, thus preventing accurate quantitation of relative activity. Deletion of the terminal 560-570 residues resulted in a complete loss of gp91^{phox} expression suggesting that the region may be important for stable expression of flavocytochrome *b*.

A separate investigation (157) of the inhibitory effects of the ⁵⁵⁹RGVHFIF⁵⁶⁵ peptide sequence was conducted utilizing a semi-recombinant reconstitution system consisting of isolated neutrophil plasma membranes and recombinant p47^{phox}, p67^{phox} and Rac1. The peptide was found to inhibit AA-mediated plasma membrane translocation of both p47^{phox} and p67^{phox}, and kinetic analyses suggested that the effects were non-competitive or mixed with respect to all of the cytosolic factors. The authors proposed that the effects of the peptide might be exerted by binding directly to and altering the conformation of flavocytochrome *b*. In contrast, a recent investigation by members of our laboratory (158) resulted in resolution of a three-dimensional structure of a peptide, corresponding to the C-terminal ⁵⁵¹SNSESGPRGVHFIFNKEN⁵⁶⁸ residues of gp91^{phox},

bound to p47^{phox} using transferred nuclear Overhauser effect spectroscopy (Tr-NOESY) NMR. Correspondingly, the majority of the contact region was found to be contained within the segment ⁵⁵⁸RGVHFIF⁵⁶⁴ indicating that this region of gp91^{phox} binds directly to p47^{phox}.

Collectively, the current data suggest that several regions of gp91^{phox} interact with the cytosolic subunits of the oxidase. These regions are therefore exposed to the neutrophil cytoplasm.

Structural and Functional Regions of p22^{phox}

Human p22^{phox} is a non-glycosylated (88), 192 amino acid residue protein (including the initiation methionine) with a predicted mass of 21.0 kDa based on primary sequence data (92). The presence of p22^{phox} is absolutely necessary for oxidase function *in vivo* as evidenced by p22^{phox} deficiencies that lead to a rare autosomal recessive form of CGD (19,28,159). p22^{phox} also appears to be necessary for stability of the flavocytochrome *b* heterodimer as the majority of CGD mutations that are confined to p22^{phox} also result in complete loss of gp91^{phox} (17,19,26,160-162).

Studies of interactions that occur between proteins that contain SH3 domains and their complimentary proline-rich target sites have identified a number of SH3-target sequence motifs including XPXXPPPΨXP (Ψ is a hydrophobic residue) (163) and PPRP (164), both of which are represented within the C-terminal ¹⁵¹PPSNPPPRPP¹⁶⁰ region of p22^{phox}. Several studies therefore, have been directed at determining if the tandem SH3

domains, that are present in both p47^{phox} and p67^{phox}, might bind to this target region of p22^{phox}. Similar to gp91^{phox}, the primary focus of research has been directed towards interactions that occur between p47^{phox} and p22^{phox}. However, a recent study conducted by Dahan *et al.*, utilizing the methodology of "peptide walking", suggested that p67^{phox} might also bind p22^{phox} (165). Ninety-one synthetic overlapping pentadecapeptides spanning the entire p22^{phox} subunit were tested for their ability to either inhibit *in vitro* oxidase activation, or when immobilized, bind to the cytosolic subunits. They were able to identify three possible regions of p22^{phox}, namely, residues ⁸¹Gly-Ala⁹¹, ¹¹¹Thr-Ala¹¹⁵, and ¹⁵¹Pro-Pro¹⁶⁰, that appeared to coordinate interactions with p67^{phox}. They also identified p22^{phox} residues ⁵¹Leu-Ser⁶³ and ¹⁵¹Pro-Pro¹⁶⁰ as binding sites for p47^{phox}, suggesting that both p47^{phox} and p67^{phox} target the same proline-rich C-terminal region of p22^{phox}.

Park *et al.* was able to inhibit *in vitro* oxidase activation using a 14 residue peptide corresponding to residues ⁸²PFTRNYYVRAVLHL⁹⁵ of p22^{phox} at an IC₅₀ concentration of ~10 μ M (166). Truncated versions of the peptide were also tested for their ability to inhibit oxidase activation, and a minimal sequence that retained inhibitory capacity (IC₅₀ value lower than 50 μ M) was found, consisting of residues ⁸⁴Thr-Leu⁹³. Single alanine replacements of the individual residues within another truncated peptide sequence, ⁸³Phe-Leu⁹³, with exception of Arg⁹⁰ which was substituted with a glutamine, suggested that the central Tyr^{87/88} and Val⁸⁹ were the most crucial residues (~ 3-4-fold increase in IC₅₀ concentrations), and to a lesser extent, Arg⁸⁵ and Val⁹² (~ 2-fold increase IC₅₀ concentrations). The oxidase inhibitory effects of this peptide are supported

experimentally by the work of Dahan *et al.* (165), who proposed that p22^{phox} residues 81-91 might mediate p67^{phox} binding.

Analysis of a CGD patient revealed normal expression levels of flavocytochrome *b* that showed no spectral abnormality, and normally functioning cytosol when tested *in vitro* with normal neutrophil membranes (167). Sequence analysis of the cDNA from the patient revealed a single-base substitution that predicted a nonconservative Pro¹⁵⁶→Gln substitution in one of the proline-rich C-terminal regions of p22^{phox}. Anti-peptide antibodies made from a synthetic peptide of the residues 153-164 of p22^{phox} were shown to bind only to permeabilized neutrophils suggesting that epitope was located on the intracellular aspect of the neutrophil membrane. Further analyses of this mutation conducted by Leusen *et al.* (128), showed that membrane translocation of both p47^{phox} and p67^{phox} was severely attenuated in PMA-stimulated neutrophils isolated from the patient. In a cell-free assay, superoxide production was negligible when neutrophil membranes from the patient were tested with normal cytosol, and membrane translocation of p47^{phox} and p67^{phox} appeared to be completely abrogated. The neutrophil membranes of the patient exhibited cytosol-independent oxygen consumption, under conditions established by Koshkin and Pick (125,126), that was similar to control neutrophil membranes suggesting that NADPH and FAD binding were not affected. Although membrane translocation of both p47^{phox} and p67^{phox} were affected by the mutation, a peptide mimicking the putative SH3 binding domains of p22^{phox} (residues 149-162), and another containing the Pro¹⁵⁶→Gln substitution, were both ineffective

($IC_{50} \sim 100 \mu M$) at inhibiting oxidase activity when tested in a cell-free assay derived from normal neutrophils.

Nakanishi *et al.* (150) was able to substantially inhibit oxygen consumption in a cell-free assay activated with SDS by addition of a peptide mimetic (designated S_C) of the C-terminal 176-195 residues of $p22^{phox}$ ($IC_{50} = 36 \mu M$). Additional experiments were conducted where cytosol and high concentrations of the peptide (100 μM) were incubated together, and then cross-linked with dimethyl 3,3'-dithiobis-propionimidate (DTBP). Immunoblots probed with $\alpha-S_C$ antibodies were positive for a 47 kDa protein that was probably $p47^{phox}$. DeLeo *et al.* (129) screened $p47^{phox}$ with a random-sequence peptide phage display library and found two consensus peptide sequences that corresponded to the putative SH3 binding regions, residues 156-160 and 177-183 of $p22^{phox}$. In contrast to the results obtained by Nakanishi *et al.* (150), a peptide mimicking the same C-terminal 176-195 residues of $p22^{phox}$ was shown to be a poor inhibitor of oxidase activation *in vitro* ($IC_{50} \sim 500 \mu M$).

In a tour de force, Leto *et al.* (168) also conducted a study aimed at identifying the oxidase protein target sites of the SH3 binding domains of $p47^{phox}$ and $p67^{phox}$. GST-fusion proteins were constructed with short peptide sequences corresponding to the SH3 domains of $p47^{phox}$ ($p47^{phox}/SH3_{A-B}$, residues 151-284; $p47^{phox}/SH3_A$, and $/SH3_B$, residues 151-214 and 227-284, respectively) and $p67^{phox}$ ($p67^{phox}/SH3_A$ and $/SH3_B$, residues 241-304 and 458-526, respectively). Two additional constructs were made, one with GST fused to the cytoplasmic domain of $p22^{phox}$ (GST- $p22^{phox}$)(residues 127-195), and another with the same residues but containing a Pro¹⁵⁶→Gln substitution (GST- $p22^{phox}\Delta$). These

p22^{phox} fusion proteins were then subjected to SDS-PAGE, transferred to nitrocellulose, and probed with affinity purified, biotinylated p47^{phox} and p67^{phox} SH3 fusion proteins. Under these conditions, the p47^{phox}/SH3_{A-B} probe bound to GST-p22^{phox} and p47^{phox}, but not GST-p22^{phox}Δ or p67^{phox}. Probing with p47^{phox}/SH3_A produced only weak signal, but probing with p47^{phox} SH3_B showed strong binding to both GST-p22^{phox} and GST-p22^{phox}Δ suggesting that the specificity of the interaction was compromised in the absence of both SH3 domains. Conversely, probing with either of the p67^{phox} constructs produced only weak signal suggesting a lower affinity for the C-terminal region of p22^{phox}, or possibly that both p67^{phox} SH3 domains were necessary for efficient binding. Several peptides representing the proline-rich regions of either p22^{phox} (residues 149-162, or 149-162 Pro¹⁵⁶→Gln, and 176-195) or p47^{phox} (residues 70-83, 338-351, and 358-371) were tested for their ability to inhibit binding interactions on the immunoblots. Peptide p22^{phox} 149-162 was the only effective inhibitor of the p22^{phox}-p47^{phox} binding interaction. Peptide p22^{phox} 176-195 was unable to inhibit binding, as was the 149-162 Pro¹⁵⁶→Gln peptide, thus corroborating the *in vitro* functional results of both DeLeo *et al.* (129) and Leusen *et al.* (128), and also confirming the biochemical effects of the CGD mutation (167). Further experiments using EBV-transformed p22^{phox} deficient B-cells expressing either wt p22^{phox} or p22^{phox} Pro¹⁵⁶→Gln (168) showed that oxidase activity could only be rescued by transfection with wt p22^{phox}. Transfection of a K562 erythroleukemia cell line with the same constructs demonstrated that translocation of p47^{phox} was completely abolished by the Pro¹⁵⁶→Gln substitution, and also that p47^{phox} translocation occurred in the absence of gp91^{phox}.

Sumimoto *et al.* (169) performed similar experiments with GST-fusion protein constructs to specifically examine the interactions between the SH3 domains of p47^{phox} and the C-terminal proline-rich region of p22^{phox}. A GST-fusion protein containing the SH3 domains (residues 154-285) of p47^{phox} (GST-p47^{phox}-SH3) was shown to inhibit superoxide activity in a cell-free assay, activated with either 50 μ M AA or 100 μ M SDS, at an IC₅₀ concentration of $\sim$ 20 nM. GST-fusion proteins were also expressed that contained the residues 132-195, 145-170, 151-160 of p22^{phox}, and two additional ones containing residues 132-195, and 151-160, but including the known CGD mutation Pro¹⁵⁶ $\rightarrow$ Gln (167). These GST-p22^{phox} protein constructs were subjected to SDS-PAGE, transferred to nitrocellulose, and then probed with biotinylated GST-p47^{phox}-SH3. GST-p47^{phox}-SH3 bound only GST-p22^{phox} proteins that were lacking the Pro¹⁵⁶ $\rightarrow$ Gln substitution, with the highest affinity interaction observed between GST-p47^{phox}-SH3 and GST-p22^{phox}-(132-195). Although GST-p22^{phox}-(151-160) contained the proline-rich, putative SH3 target sequence, binding to this region by GST-p47^{phox}-SH3 was considerably diminished, suggesting that the structure of the SH3 target region was compromised, or possibly that SH3-mediated binding is only partly responsible for the interprotein coordination.

A later study conducted by the same group (170) investigated which of the p47^{phox} SH3 domains bound to p22^{phox}, and also which of the specific proline sequences of p22^{phox} was responsible for the interaction. Using the same system, they expressed GST-p47^{phox} fusion proteins containing full length p47^{phox} (p47^{phox}-F, residues 1-390), p47^{phox} SH3(N) (residues 154-219, and 154-219 with a Trp¹⁹³ $\rightarrow$ Arg substitution), p47^{phox}

SH3(C) (residues 223-286), and p47^{phox}(SH3)₂ (residues 154-286). GST-p22^{phox} fusion proteins were also expressed that contained residues 132-195, 132-195 with Pro¹⁵⁶→Gln substitution, 132-150, 132-170, 145-170, 145-195, and 163-195. Immunoblots of the GST-p22^{phox}-132-195 fusion protein were probed first with the various GST-p47^{phox} fusion proteins, and then an α-GST monoclonal antibody. Strong binding was evident between p47^{phox} SH3(N) and GST-p22^{phox}-132-195, but was completely abolished with GST-p22^{phox}-132-195 Pro¹⁵⁶→Gln indicating that the region represented at least one target for the SH3-mediated interactions. The mutant p47^{phox} SH3(N)-(154-219, Trp¹⁹³→Arg) also failed to bind the GST-p22^{phox}-132-195 suggesting that p47^{phox} SH3(N) binds p22^{phox} in a manner common among SH3 binding proteins (171,172) where a tryptophan residue interacts directly with a proline. Immunoblots of GST-fusion p22^{phox} proteins probed with p47^{phox}-SH3(N) showed strong binding to GST-fusion p22^{phox} proteins that contained the stretch ¹⁵²PSNPPPRPP¹⁶². In contrast, GST-fusion p22^{phox} proteins that lacked this stretch (residues 132-150, and 163-195) did not interact with p47^{phox}-SH3(N) indicating that the ¹⁵²PSNPPPRPP¹⁶² region was the target for the SH3 regions of p47^{phox}. These results were confirmed *in vivo* using two separate yeast two-hybrid systems where productive interactions were observed only in cells that coexpressed both p22^{phox}-(132-195) and p47^{phox}(SH3)₂ (residues 154-286). GST-p22^{phox}-132-195 immobilized on a biosensor tip in a resonance mirror system (173-175) was also shown to directly bind GST-p47^{phox} SH3(N) or GST-p47^{phox}(SH3)₂ (respective K_ds of 0.34 and 0.36μM), but not GST-p47^{phox} SH3(N) containing the Trp¹⁹³→Arg substitution, or GST-p47^{phox}-SH3(C). Lastly, GST-p47^{phox}-F was able to reconstitute full activity in a

cell-free superoxide assay in the presence of anionic activators, whereas GST-p47^{phox}-F containing the Trp¹⁹³→Arg substitution was inactive. These findings suggest that the SH3(N) domain of p47^{phox} (residues 154-219) binds specifically to the C-terminal proline-rich region (residues ¹⁵²PSNPPPRPP¹⁶²) of p22^{phox}.

In similar experiments (176), a truncated form of p47^{phox} that was lacking the C-terminal Arg/Lys regions (p47^{phox}-ΔC, residues 1-286) was precipitated by a maltose binding protein (MBP) fusion construct containing p22^{phox} residues 132-195 (MBP-22-C), but not by MBP-22-C carrying the Pro¹⁵⁶→Gln substitution. MBP-22-C was also able to precipitate full-length GST-p47^{phox}-F (residues 1-390) substitution mutants containing Pro^{229/300}→Gln, Arg^{301/302}→Glu, and Ser^{303/304/328}→Asp, but not wt GST-p47^{phox}-F, or p47^{phox}-F substitution mutants carrying Ser³²⁸→Asp, or Ser^{303/304}→Asp replacements. To further test the hypothesis that disruption of the intramolecular interactions within p47^{phox} were necessary to promote accessibility of the SH3 domain to p22^{phox}, they conducted *in vivo* experiments using a Y190 yeast two-hybrid system. Positive binding interactions were observed only in cells that were co-transformed with p22^{phox}-C and either p47^{phox}-ΔC, or p47^{phox}-(1-302), but not wt p47^{phox}-F. In the same system, p47^{phox}-F double mutants with Pro^{299/300}→Gln, or Arg^{301/302}→Glu substitutions, were both able to bind p22^{phox}-C. However, under the same conditions, a p47^{phox}-F double mutant with aspartate substitutions at Ser^{303/304}→Asp failed to interact with p22^{phox}-C, thus consistent with observations from their precipitation experiments. C-terminally truncated versions of p47^{phox} (residues 1-327 or 1-340), both with the double

Ser^{303/304}→Asp substitution were also tested in the yeast two-hybrid assay. The p47^{phox}-(1-327), Ser^{303/304}→Asp was able to bind p22^{phox}-C, but inclusion of a third Ser³²⁸→Asp substitution within the p47^{phox}-(1-340) truncated mutant, and also into the p47^{phox}-F conferred positive binding activity, whereas the single Ser³²⁸→Asp substitution was inactive. Additional p47^{phox}-F substitution mutants were tested, where serines 303, 304, 315, 320, 328, 345, 348, 359, 370, and 379 were all simultaneously replaced with aspartate, or all but serines 303, 304, or 328 were replaced. Only the mutant p47^{phox}-F that contained simultaneous aspartate substitutions at serines 303, 304, and 328 could bind p22^{phox}-C, suggesting that these residues were critical for intramolecular masking of the SH3 domain that binds p22^{phox}.

Since the GST-p47^{phox}-ΔC mutant was previously shown able to initiate a limited amount of amphiphile-independent superoxide production *in vitro* (177,178), GST-p47^{phox}-F proteins, wt or substitution mutants (Pro^{299/300}→Gln, Arg^{301/302}→Glu, Ser^{303/304}→Asp, Ser³²⁸→Asp, Ser^{303/304/328}→Asp), were also tested for their ability to reconstitute superoxide activity *in vitro* in the presence or absence of SDS. Oxidase activation using wt GST-p47^{phox}-F was absolutely dependent on the presence of SDS. In the absence of SDS, GST-p47^{phox}-F mutants Pro^{299/300}→Gln, Arg^{301/302}→Glu, and Ser^{303/304/328}→Asp were all capable of reconstituting *in vitro* oxidase activity at ~ 50% of the amphiphile-activated system, but at a ~ 5-8 fold higher concentration relative to GST-p47^{phox}-ΔC. The triply mutated GST-p47^{phox} (Ser^{303/304/328}→Asp) was the least active, requiring the highest concentration (~ 400 nM) to achieve oxidase activity close to

p47^{phox}-ΔC (~ 50 nM). All of the mutant GST-p47^{phox} tested showed similar activity in the presence of SDS. The ability of the different mutants to reconstitute oxidase activation *in vitro* corresponded identically to their ability to bind p22^{phox} shown in their previous experiments. These results suggest that the rate-limiting step in oxidase activation *in vitro* in their system was dependent on p47^{phox} binding to p22^{phox}. The effects of these p47^{phox} mutations were further investigated *in vivo* using transfected K562 cells, where serines 303, 304, and 328 were replaced with alanine, a residue that cannot be phosphorylated. Cells transfected with wt p47^{phox}-F were capable of full oxidase activity, whereas cells expressing mutant p47^{phox}-F containing either Ser³²⁸→Ala or Ser^{303/304}→Ala substitutions showed very little oxidase activity even though similar amounts of p47^{phox} were expressed. Collectively, these results suggest that simultaneous phosphorylation of p47^{phox} serines 303, 304, and 328 is necessary for disruption of intramolecular binding interactions within p47^{phox}. Once disrupted, the SH3 domains of p47^{phox} are unmasked, thereby allowing interaction with the C-terminal regions of p22^{phox} and subsequent activation of the oxidase.

Huang and Kleinberg (132) used a *Saccharomyces cerevisiae* yeast two-hybrid system to study the interactions between the SH3 (p47^{phox}-NTSH3_{A-B}, residues 1-285; p47^{phox}-SH3_{A-B}, residues 154-285; p47^{phox}-SH3_A, and -SH3_B, residues 146-228 and 222-285, respectively) and the Arg/Lys (residues 286-351) domains of p47^{phox}, with the C-terminal region of p22^{phox} (p22^{phox}-CT, residues 135-195). Again, the role of the Pro¹⁵⁶ of p22^{phox} was investigated by incorporating a Pro¹⁵⁶→Gln substitution into the C-terminal region of p22^{phox} (p22^{phox}-*CT). Additionally, full length p47^{phox}, either wt or mutated,

were expressed as either B42 or LexA activation domain fusion proteins in a yeast two-hybrid system. In the yeast two-hybrid binding assay, p47^{phox}-SH3_A and p47^{phox}-SH3_{A-B}, but not p47^{phox}-SH3_B, bound p22^{phox}-CT, and none of the p47^{phox} constructs bound to p22^{phox}-*CT. Unexpectedly, coprecipitation of p47^{phox}-SH3_{A-B} by immobilized GST-p22^{phox}-CT, could also be blocked by synthetic p47^{phox} Arg/Lys domain peptides corresponding to residues 301-320 and 314-335 even though these peptide regions did not bind to p22^{phox}-CT in the yeast two-hybrid assay. These peptide regions, when expressed as GST-p47^{phox} fusion proteins, also failed to coprecipitate GST-p22^{phox}-CT. Further investigation, using the yeast two-hybrid system, showed that wt p47^{phox} did not interact with p22^{phox}-CT. To investigate whether p47^{phox} Arg/Lys domain binding to the SH3_{AB} domain could be reversed by conditions mimicking phosphorylation, or anionic amphiphile interaction, aspartate or alanine substitutions were incorporated within the Arg/Lys domain of wt p47^{phox} at Ser³¹⁰ and Ser³²⁸. Serine replacement, either separately or in tandem, with alanine or aspartate was intended to mimic neutral or negative charge, respectively. Positive binding interactions with p22^{phox}-CT, but not p22^{phox}-*CT, were observed for the both single and double Ser³²⁸→Asp substitutions, and also with the single Ser³¹⁰→Ala substitution. These results indicated that a single, negatively-charged residue within the Arg/Lys domain was sufficient to disrupt the intraprotein binding between the Arg/Lys and the SH3_{AB} domains of p47^{phox}, and that the interaction might be stabilized by the hydroxyl side group of Ser³¹⁰. Superoxide generation *in vitro* with wt p47^{phox} required addition of arachidonate, whereas similar amounts of activity were achieved with p47^{phox}-SH3_{AB} that showed no dependence on arachidonate. Their results

suggested that the p47^{phox} Arg/Lys domains interact preferentially with the SH3 domains of p47^{phox}, and block the SH3_A domain of p47^{phox} from binding the C-terminal, proline-rich region of p22^{phox}. They hypothesized that, *in vivo*, incorporation of a negative charge by serine phosphorylation within the Arg/Lys domain of p47^{phox} could disrupt intramolecular interactions with the SH3 domains, thereby enabling binding between SH3_A and the C-terminal proline-rich region of p22^{phox}. In support of this hypothesis, expression of a double mutant p47^{phox} containing alanine substitutions at serines 303 and 304 was unable to restore normal oxidase activity in an EBV-transformed p47^{phox}-deficient cell line (179). However, transfection with a double mutant p47^{phox} containing either glutamate or lysine substitutions of the same serines was able to rescue oxidase activity, thus contrasting the results of Ago *et al.* (176).

Interactions Between gp91^{phox} and p22^{phox}

The specific residues that form the interfacial contact region between p22^{phox} and gp91^{phox} have not been identified. Recent proteolysis experiments conducted by our group (140) resulted in isolation of a spectrally stable core polypeptide comprising the NH₂-terminal 320 to 363 amino acid residues of gp91^{phox}, and the NH₂-terminal 169 to 171 amino acid residues of p22^{phox}. The overall stability of the polypeptide, as evidenced by retention of a native Soret absorbance spectrum, suggested that the gp91^{phox}-p22^{phox} interprotein contact residues were contained within this core polypeptide.

Cofactor Binding Regions of Flavocytochrome *b*.

NADPH

Early attempts at identification of the NADPH-binding component(s) of the oxidase using affinity labeling with NADPH analogues produced conflicting reports of membrane bound proteins of 66 (180,181) and 32 kDa (182), and one 66 kDa cytosolic protein (183) that may have been p67^{phox} (184,185). The first compelling evidence that gp91^{phox} could bind NADPH was provided by Segal and coworkers using the NADPH analogue, ³²P-labeled 2-azido-NADP (114). Labeling with this derivative was positive for gp91^{phox} derived from normal neutrophils, but negative when tested against neutrophil membranes of X91⁰ CGD patients. Confirmation of NADPH binding to gp91^{phox} was provided by later experiments conducted by separate groups using other NADPH analogues such as the tritiated NADPH derivative, [4-[N-(4-azido-2-nitrophenyl)[³H]amino]butyryl]NADPH ([³H]azido-NADPH) (122), and also by reduction of the schiff base formed between an NADPH analogue, pyridoxal-5'-diphospho-5'-adenosine (186), and gp91^{phox} with Na[³H]BH₄ (123). Han *et al.*, using a recombinant bacterial expression system to produce truncated forms of gp91^{phox}, was able to further localize the NADPH binding domain to the C-terminal 306-569 amino acid residues of the protein (146).

Details of the specific gp91^{phox} residues that might coordinate NADPH have been inferred by sequence comparison to NADPH binding domains of other flavoproteins such as porcine and human NADH-cytochrome *b5* reductase, spinach ferredoxin-NADP+ reductase (FNR), ferric reductase of *Saccharomyces cerevisiae* (FRE1), rat nitric oxide synthase (NOS), NADPH-cytochrome *P450* reductase (CPR) from *Bacillus megaterium* and rat, tomato NADH-nitrate reductase, NADPH-sulfite reductase, and the human gp91^{phox} homologue, p65^{phox} (113-115,124,187-197). A consensus NADPH-binding motif was found, composed of a glycine-rich stretch, GXGXGXXPF, in tandem with a separate CG couplet. This motif is present in the C-terminal regions of gp91^{phox} corresponding to residues ⁴⁰⁸GAGIGVTPF⁴¹⁶, and ⁵³⁴VFXCGP⁵³⁹.

Several CGD case studies provide indirect support for these regions as NADPH binding sites. An X91⁺ CGD case was found to be caused by a single Leu⁵⁴⁶→Pro substitution in gp91^{phox} (198). The authors proposed that inclusion of a Pro residue could severely perturb the putative α -helical structure that was predicted for this region of gp91^{phox} (197), and thus affect NADPH binding. In a separate X91⁺ CGD case, arising from a single Pro⁴¹⁵→His point mutation in gp91^{phox} (199), the physicochemical properties of the flavocytochrome *b* heme from the patient appeared normal with respect to absorbance spectrum, redox potential and CO binding (200). Although normal amounts of flavocytochrome *b* were present in the neutrophil membranes of this patient, labeling with the NADPH analogue ³²P-labeled 2-azido-NADP was abolished (114). The same mutation was found in another CGD patient (201). Biochemical analyses of neutrophils from the patient showed that the flavocytochrome *b* was able to bind normal

amounts of FAD, but both INT, and NBT diaphorase activity was entirely absent suggesting that electrons could not pass from NADPH to FAD. Together, these studies suggest that the Pro⁴¹⁵→His mutation affects binding of NADPH.

In a separate CGD case study (124), a mutation causing a Gly⁴⁰⁸→Glu substitution in gp91^{phox} was found. Although this mutation resides within the putative binding region of the pyrophosphate moiety of NADPH (202), it is interesting to note that translocation of both p47^{phox} and p67^{phox} was completely abolished in cell-free assays, and also in intact neutrophils of the patient, even though substantial amounts of both flavocytochrome *b* subunits were present. Another study of a 69 year old X-91⁺ terminally ill CGD patient (203) revealed a splice junction mutation that resulted in a deletion of residues 488-497 of gp91^{phox} (Δ 488-497 gp91^{phox}). To characterize the functional consequences of this mutation, Yu *et al.* (204) expressed this mutant form of flavocytochrome *b* in a gp91^{phox}-negative PLB-985 cell line (156) by transfection with Δ 488-497 gp91^{phox} cDNA. Translocation of p47^{phox} and p67^{phox} was not affected by the mutation in either the cell-free assay or intact cells, and a peptide corresponding to gp91^{phox} residues 488-497 was only a weak inhibitor of superoxide production *in vitro* (IC₅₀ = ~ 500 μ M). The Δ 488-497 gp91^{phox} was also able to bind normal amounts of FAD, but was unable to reduce INT indicating that electron transfer was disrupted prior to reduction of FAD. These results suggested that either NADPH was unable to bind the flavocytochrome *b*, or that the deficiency was due to impaired delivery of electrons from NADPH to FAD. Another CGD case study found a mutation close to this region that gave rise to a replacement of the three residues ⁵⁰⁷GKT⁵⁰⁹ of gp91^{phox} with the four

residue sequence, ⁵⁰⁷HIWA⁵¹⁰ (205). Neutrophils isolated from the patient were unable to produce detectable amounts of superoxide, but the membrane fraction was able to sustain cell-free oxidase activity at ~ 7% of control membranes with cytosolic fractions derived from either the patient or normal controls. Flavocytochrome *b* expression levels appeared normal, and membrane translocation of either p47^{phox} or p67^{phox} did not appear to be affected suggesting that the deficiency resided within the electron transport chain. The authors proposed that the substitutions occurred close enough to the putative NADPH binding regions of gp91^{phox} to perturb binding, though actual NADPH binding assays were not conducted. These reports, in conjunction with the modeling studies, suggest that the ⁴⁰⁸GAGIGVTPF⁴¹⁶, and ⁵³⁴VFXCGP⁵³⁹ residues of gp91^{phox} are likely to define the respective contact sites of the ribose and adenine moieties of NADPH.

FAD

The FAD binding regions of flavocytochrome *b* have also been localized to gp91^{phox} by applying techniques similar to those used for NADPH. Gp91^{phox} has been photoaffinity labeled directly using a tritiated, 4-[N-(4-azido-2-nitrophenyl)amino]butyryl-FAD, ([³H]NAP4-FAD) (111), 5-deaza-FAD (121), and most recently with a fluorescent analogue, thioacetamido-FAD (8-nor-8-S-thioacetamido-FAD) (206). As with NADPH, Han *et al.* (146) concluded that the FAD binding region was also contained within the C-terminal 307-570 residues of gp91^{phox}.

The specific residues of gp91^{phox} that might coordinate FAD have been inferred from primary sequence similarities to FAD binding regions of other proteins (115), primarily flavoproteins of the ferredoxin-NADP⁺ reductase family (114,197). The prototypical structural elements of this family derive from the X-ray diffraction crystallographic structure of cofactor-bound spinach ferredoxin-NADP⁺ reductase (207). The residues ³³⁸HPFTLTS³⁴⁴ of gp91^{phox} correspond to an FAD-binding motif, HPFTXXS that is strictly conserved in within a group of the yeast ferric reductases. (94,197), and also in porcine (208) and mouse (209) gp91^{phox}. Biochemical support for these inferences has been provided by mutational analyses of several CGD cases. The neutrophil membranes of an X91⁻ CGD patient with a point mutation resulting in a His³³⁸→Tyr substitution were entirely depleted of FAD (117,210). This same His³³⁸→Tyr mutation was observed in separate CGD case, as was a Pro³³⁹→His substitution in another (198). These two patients exhibited the same X91⁻ phenotype (~30-40% of normal controls), and an impaired hydrogen peroxide production that was disproportionately low for the amount of flavocytochrome *b* present in the membranes. The authors speculated that the decreased oxidase activity was due to a lack of bound FAD or impaired binding based solely on modeling studies. Mutational analyses of two newly identified X91⁺ CGD patients revealed substitutions at Thr³⁴¹→Lys, and Cys³⁶⁹→Arg in gp91^{phox} (124). Cell-free oxidase studies indicated that both patients had normal functioning cytosol, thus localizing the deficiency to the membrane fraction. The Thr³⁴¹→Lys patient showed normal cytosol to membrane translocation of p47^{phox} and p67^{phox} in both intact PMA-stimulated neutrophils, and in the cell-free assay. However,

the neutrophil membranes of the patient showed no oxygen consumption under conditions established by Koshkin and Pick (125,126). Since the mutation resided within the putative binding region of the isoalloxazine moiety of FAD (114), the authors hypothesized, by analogy to mutagenesis studies of FNR (211) and flavin oxidoreductase (212), that the loss of oxidase activity was due to impaired hydride transfer between NADPH and FAD. The Cys³⁶⁹→Arg substitution of the other patient is situated within a region predicted to be important for interaction with the pyrophosphate moiety of the FAD. Neutrophil membranes from this patient were capable of only 3% of the oxygen consumption of normal membranes, but with correspondingly low translocation of equal amounts of p47^{phox} and p67^{phox} in both PMA-stimulated intact neutrophils and cell-free assays. Three separate CGD cases that were studied by Bu-Ghanim *et al.* (213), revealed gp91^{phox} mutations that resulted in a Cys²⁴⁴→Tyr substitution, a single deletion of one of the three lysines, Lys³¹³⁻³¹⁵, and a premature stop codon causing a deletion of the C-terminal six residues of gp91^{phox} (Δ565-570). The authors speculated that the deficiencies of the Δ565-570 and the lysine deletion could be due to impaired cofactor binding based on the structural model proposed by Segal *et al.* (197).

Anti-flavocytochrome *b* Antibodies.

Information derived from flavocytochrome *b*-specific antibodies has provided insight into the structural topology of flavocytochrome *b* and in some cases, function of the oxidase. The currently available information, either published previously, or from

ongoing work performed in our laboratory, is listed in Tables 1.1 and 1.2 where their characteristics are summarized. The antigen and the animal used to produce the antibodies is also listed as is the type of antibody (i.e. monoclonal or polyclonal). The information is divided into α -gp91^{phox} and α -p22^{phox} antibodies, and organized relative to the primary sequence of each subunit beginning at the NH₂-terminal regions.

Imajoh-Ohmi *et al.* conducted a study of the topology of flavocytochrome *b* using a combination of four α -flavocytochrome *b* antibodies and proteolysis (214). Two of the antibodies were prepared from synthetic peptides corresponding to p22^{phox} residues 2-26 and 176-195 (designated S_N and S_C, respectively), and two others were prepared from synthetic peptides mimicking gp91^{phox} residues 151-173 and 551-570 (designated L₁₂₃ and L_C, respectively). Flow cytometrical analyses using antibodies S_N and S_C comparing intact to freeze-thaw permeabilized neutrophils indicated an intracellular location for both S_N and S_C epitopes. Exposure of intact neutrophil cytoplasts to papain, bromelain, subtilisin, trypsin, and proteinase from *Streptomyces caespitosus* had no effect on p22^{phox}. Conversely, treatment of cytoplasmic membranes with the same proteases resulted in removal of the C-terminal region of p22^{phox}. Interestingly, an NH₂-terminal ~ 10 kDa region of p22^{phox} was shown to form a protease-resistant structure that vanished once the protein was solubilized in Triton X-100. These observations suggested that the NH₂-terminal region of p22^{phox} forms a structure that is buried in the membrane, or possibly bound to gp91^{phox} in such a way that it is inaccessible to protease. Antibodies L₁₂₃ and L_C were also used for flow cytometrical analyses comparing intact to permeabilized neutrophils. The L₁₂₃ antibody bound intact neutrophils whereas the L_C antibody was

positive only on permeabilized neutrophils indicating extra- and intracellular epitope locations, respectively. Digestion profiles from intact cytoplasts by immunoblot analyses revealed an 18 kDa fragment of gp91^{phox} which retained the region ¹⁵¹Ser-Leu¹⁷³. The fragment accumulation paralleled increasing concentrations of papain indicating that gp91^{phox} possessed a papain-sensitive site on the extracellular aspect of the cells. The authors proposed that the extracellular papain-sensitive region was probably contained within the residues ³⁷⁰Gly-Phe³⁹⁹ of gp91^{phox} based on mass analysis by SDS-PAGE, flow-cytometry, and hydropathy data.

The first report of a flavocytochrome *b*-specific mAb was that of mAb 7D5 by Nakamura *et al.* (215). Although mAb 7D5 was initially believed to bind the extracellular aspect of p22^{phox} (216), later work by the same group (217) and also by us (218) concluded that the target epitope is accessible on the extracellular aspect of native gp91^{phox} of primate origin (217). The residues defining the epitope of mAb 7D5 were determined by our group using a random-nonapeptide phage display library (218). The epitope was mapped to two discontinuous linear stretches of gp91^{phox} consisting of residues ²²⁶RIVRG²³⁰ and ¹⁶⁰IKNP¹⁶³. These sequence stretches are thus restricted to the extracellular aspect of the neutrophil membrane, and are positioned adjacent to each other in the native structure. Similar structural information was obtained by our laboratory using the p22^{phox}-specific mAb 44.1 (219,220), and also the gp91^{phox}-specific mAb 54.1 (220). The phage sequences selected by mAb 54.1 suggest that the antibody binds to the ¹⁸¹GGPQVNPI¹⁸⁸ region of gp91^{phox} (220). Under ELISA conditions, mAb 54.1 was able to bind both detergent-solubilized flavocytochrome *b* and flavocytochrome *b* in

neutrophil membrane vesicles. MAb 54.1 was also able to immunosediment detergent-solubilized flavocytochrome *b*, but for reasons that are not clear, was not able to bind gp91^{phox} in either intact or permeabilized neutrophils.

Similar to mAb 7D5, the mAb 44.1 epitope was also determined to be composed of two discontinuous linear regions of p22^{phox}, consisting of residues ²⁹TAGRF³³ and ¹⁸¹GGPQVNPI¹⁸⁸, that resided on the intracellular aspect of the neutrophil membrane (219,220). A synthetic contiguous peptide construct, consisting of these two stretches of residues linked by an additional glycine, was used in conjunction with mAb 44.1 to investigate the bound structure of the peptide using transferred NOESY and transferred ROESY NMR (219). This approach provided an "antibody imprint" of the surface topology of the mAb 44.1 epitope region. The results suggested that the bound peptide formed a folded conformation thus inferring that the separate epitope regions of p22^{phox}, separated by putative membrane-spanning domains, were juxtaposed in the native protein.

Two monoclonal antibodies, mAb 48 and mAb 449, were prepared by Verhoeven *et al.* (160) by injecting mice with purified, Triton X-100 solubilized flavocytochrome *b*. MAb 48 was determined to be specific for gp91^{phox} and mAb 449 was specific for p22^{phox}. Flow cytometric analysis comparing intact to permeabilized neutrophils indicated that the mAb 449 epitope was located on the cytosolic aspect of the plasma membrane. MAb 48 was positive only for denatured flavocytochrome *b* by immunoblot. These two mAbs were further characterized by our laboratory using random-peptide phage display analysis and "peptide walking" (221). These separate approaches provided

similar results suggesting that mAb 449 binds a minimum linear sequence of p22^{phox} encompassing residues ¹⁸²GPQV¹⁸⁵, and that MAb 48 binds a linear sequence encompassing gp91^{phox} residues ⁴⁹⁸EKDVTGL⁵⁰⁵. The lack of immunoreactivity of mAb 48 to membrane-bound flavocytochrome *b* implies that the epitope region is inaccessible in the membrane-resident native protein.

The technology of epitope mapping α -flavocytochrome *b* antibodies using random peptide phage display libraries is presently being applied by our laboratory to characterize several of the mAbs listed below. Within this group, some are able to bind functionally important regions of flavocytochrome *b*, as evidenced by their ability to inhibit *in vitro* oxidase activity (J.B. Burritt, unpublished), while others aid in defining topological features of flavocytochrome *b*.

Table 1.1

α -gp91 ^{phox} Antibody	Ig Isotype and (κ) or (λ)	Epitope	Innoculum	In, Ex	Immunoblot	ELISA/ L, D	References
MGN (P)	NA	¹ MGNWAVNE ⁸	peptide ¹ Met-Glu ⁸ -(C)	Ex	(-)	+D	Unpublished
YRV (P)	NA	³⁰ YRVYDIPPKFFYTRK ⁴⁴	peptide ³⁰ Tyr-Lys ⁴⁴ -(C)	(-)	(-)	+D	Unpublished
CL10 (M)	G1(κ)	¹³⁵ DPYSVA ¹⁴⁰	whole flavocytochrome b	(-)	(+)	+D	Unpublished
CL11 (M)	G1(κ)	¹³⁵ DPYSVA ¹⁴⁰	whole flavocytochrome b	(-)	(+)	+D	Unpublished
CL12 (M)	G1(κ)	¹³⁵ DPYSVA ¹⁴⁰	whole flavocytochrome b	(-)	(+)	+D	Unpublished
NL10 (M)	G1(κ)	¹³⁵ DPYSVAL ¹⁴¹	whole flavocytochrome b	(-)	(+)	+D	(140)
CL5 (M)	G1(κ)	¹³⁵ DPYSVALSELGDR ¹⁴⁷	whole flavocytochrome b	(-)	(+)	+D	(140)
ESY (P)	NA	¹⁵⁰ ESYLNFAKR ¹⁵⁹	peptide ¹⁵⁰ Glu-Arg ¹⁵⁹ -(C)	Ex	(+)	+D	Unpublished
L ₁₂₃ (P)	NA	¹⁵¹ SYLNFAKRIKNPEG GLYLAVTL ¹⁷³	peptide ¹⁵¹ Ser-Leu ¹⁷³	Ex	(+)	NT	(214)
ARK (P)	NA	¹⁵⁶ ARKRIKNPEGG ¹⁶⁶	peptide ¹⁵⁶ Ala-Gly ¹⁶⁶ -(C)	Ex	(+)	+D	Unpublished
7D5 (M)	G1(κ)	²²⁶ RIVRG ²³⁰	whole flavocytochrome b	Ex	(-)	+D	(215-218)
KIS (P)	NA	²⁴⁷ KISEWGKIKEC ²⁵⁷	peptide ²⁴⁷ Lys-Cys ²⁵⁷	Ex	(+)	(+D)	Unpublished
54.1 (M)	NA	³⁸³ PKIAVDGP ³⁹⁰	whole flavocytochrome b	(-)	(+)	+L, +D	(56,220)
48 (M)	G1(κ)	⁴⁹⁸ EKDVITGL ⁵⁰⁵	whole flavocytochrome b	(-)	(+)	-L, -D	(160,221)
NL7 (M)	G1(κ)	⁴⁹⁸ EKDVITGRKQ ⁵⁰⁷	whole flavocytochrome b	In	(+)	+L, +D	(140)
KQS (P)	NA	⁵⁴⁸ KQSIENSESGPRGC ⁵⁶¹	peptide ⁵⁴⁸ Lys-Pro ⁵⁶¹	(-)	(+)	(-)	(222,223)
L _C (P)	NA	⁵⁵¹ ISNSESGRGVHFIFN KENF ⁵⁷⁰	peptide (C)- ⁵⁵¹ Ile-Phe ⁵⁷⁰	In	(+)	NT	(214)
No name (P)	NA	⁵⁵⁸ PRGVHFIFNKENF ⁵⁷⁰	peptide (CSN)- ⁵⁵⁸ Pro-Phe ⁵⁷⁰	In	(+)	NT	(141,152)
CL7 (M)	G1(κ)	?	whole flavocytochrome b	?	(+)	(+)	Unpublished

M, P Monoclonal (mouse), Polyclonal (rabbit)

Ig Immunoglobulin

* Discontiguous linear epitopes

** Conformational epitope

In, Ex

 κ , λ Kappa or Lambda light chain

L Membrane-lipid bound flavocytochrome b

D Detergent-solubilized flavocytochrome b

NT Not tested

(-) Epitope not detected

? Unknown

NA Not applicable

Table 1.2

α -p22 ^{phox} Antibody	Ig Isotype and (κ) or (λ)	Epitope	Innoculum	In, Ex	Immunoblot	ELISA L, D	References
S _N (P)	NA	² GQIEWAMWANEQAL ASGLILITGGI ²⁶	peptide ² Gly-Ile ²⁶	In	(+)	NT	(214)
44.1 (M)	G2a(κ)	^{*29} TAGRF ³³ – ¹⁸³ PQVNPI ¹⁸⁸	whole flavocytochrome <i>b</i>	In	(+)	(+)	(219,220)
NS5 (M)	G1(κ)	^{*58} KRKKGSTME ⁶⁶ – ⁷⁸ KLFGPF ⁸³	whole flavocytochrome <i>b</i>	(-)	(+)	(+)	(140)
NS2 (M)	G1(κ)	¹³¹ WTPIEPKPR ¹³⁹	whole flavocytochrome <i>b</i>	In	(+)	(+)	(140)
p22-153	NA	¹⁵³ SNPPPRPPAE ARKKPSEE ¹⁷⁰	Peptide ¹⁵³ Ser-Glu ¹⁷⁰	In	(+)	NT	(167)
EAR (P)	NA	¹⁶² EARKKPSEEEAAA ¹⁷⁴	peptide ¹⁶² Glu-Ala ¹⁷⁴	?	(+)	?	Unpublished
CS9 (M)	G1(κ)	¹⁶⁵ KKPSE ¹⁶⁹	whole flavocytochrome <i>b</i>	In	(+)	(+)	(140)
S _C (P)	NA	¹⁷⁶ AGGPPGGPQVNPPI VTDEVV ¹⁹⁵	peptide (C)- ¹⁷⁶ Ala-Val ¹⁹⁵	In	(+)	NT	(214)
449 (M)	G1	¹⁸² GPQV ¹⁸⁵	whole flavocytochrome <i>b</i>	In	(+)	+L, +D	(160,221,224)
CS8 (M)	G1(κ)	¹⁸⁵ VNPI ¹⁸⁸	whole flavocytochrome <i>b</i>	In	(+)	(+)	Unpublished
CS6 (M)	G1(κ)(λ)	¹⁸⁵ VNPI ¹⁸⁹	whole flavocytochrome <i>b</i>	In	(+)	(+)	Unpublished
NS1 (M)	G1(κ)	**GIRSXDDSG	whole flavocytochrome <i>b</i>	In	(+)	(+)	(140)
NS11 (M)	G1(κ)	**GIRSXDDSG	whole flavocytochrome <i>b</i>	In	(+)	(+)	Unpublished
R3179 (P)	NA	Unknown	whole flavocytochrome <i>b</i>	NT	(+)	NT	(88)
NS8 (M)	G1(κ)	Unknown	whole flavocytochrome <i>b</i>	In	(+)	(+)	Unpublished

M, P Monoclonal (mouse), Polyclonal (rabbit)

Ig Immunoglobulin

* Discontiguous linear epitopes

** Conformational epitope

In, Ex

 κ , λ

L

D

Intracellular, extracellular

Kappa or Lambda light chain

Membrane-lipid bound flavocytochrome *b*Detergent-solubilized flavocytochrome *b*

NT Not tested

(-) Epitope not detected

? Unknown

NA Not applicable

The Hemes of Flavocytochrome *b*.

The Number of Hemes.

Flavocytochrome *b* was initially classified as a *b*-type cytochrome based on its ferrous absorption spectrum (225), and the intrinsic heme designated as a protoporphyrin IX from its pyridine hemochrome spectrum (226). Early potentiometric titration data indicated single redox midpoint potentials for flavocytochrome *b* of -218 mV (226), -225 mV (227), -245 mV (87), -248 mV (228), -289 mV (200), and -255 mV (229), suggesting a ratio of one heme per polypeptide. The initial protoporphyrin IX classification was later confirmed by resonance Raman spectroscopy (200), but the measured heme concentration of partially-purified preparations of flavocytochrome *b* suggested a molar stoichiometry of greater than one heme per mole of cytochrome (88,227). Additional experimental evidence supporting a multiple-heme motif was contributed by redox titration data of flavocytochrome *b* from a CGD patient with a point mutation in gp91^{phox} that indicated the presence of two nonidentical hemes with midpoint potentials of -220 and -300 mV (102). Additionally, these investigators reevaluated their original redox titration data from which they had calculated a single redox potential (87,228), and concluded that there were probably two hemes with closely spaced midpoint potentials of -225 and -265 mV (102). Potentiometric titration data from recombinantly expressed gp91^{phox} also suggested the presence of multiple hemes with reduction potentials of -233 and -264 mV (230). Both flavocytochrome *b* subunits were shown to remain associated

with heme following denaturation and separation by low temperature lithium dodecyl sulfate-PAGE (103,231), and CD spectra from what was believed to be the purified small subunit of flavocytochrome *b*, showed a bilobed distribution that was interpreted as a possible heme-heme interaction (232). Additionally, low temperature absorption spectra taken from stimulated, intact porcine neutrophils showed a distinct splitting in the α -band absorbance spectrum suggesting dual heme oxidation states (233). Detergent-solubilized flavocytochrome *b*, that provided sufficient signal for analysis by resonance Raman spectroscopy, lacked a ferric EPR spectrum (200), possibly due to spin pairing between heme dimers, a conclusion that was later challenged (234). Additionally, Soret excitation and subsequent photoreduction of flavocytochrome *b* in specific granules, membrane fragments or resting neutrophils, displayed a clearly biphasic nature, possibly due multiple hemes (200). Reduced, partially-purified bovine flavocytochrome *b*, also exhibited a distinctly biphasic susceptibility of the heme to the iodonium salts, diphenylene iodonium (DPI), and iodonium biphenyl (IBP), consistent with the presence of two hemes (105). Recently, coworkers in our laboratory measured a ratio of two hemes per gp91^{phox}-p22^{phox} heterodimer in immunoaffinity-purified flavocytochrome *b* from neutrophil membranes solubilized in dodecyl maltoside (DDM) (Taylor *et al.*, unpublished). Lastly, an intramembrane, bis-heme motif within gp91^{phox} has been inferred based on similarities in primary sequence, spectral properties, and redox potential, between flavocytochrome *b* and select members of the ferredoxin-NADP⁺ reductase (FNR) family, including FRE1 ferric reductase of *Saccharomyces cerevisiae* (94,196,197,235).

Heme Ligation

The ligation of the flavocytochrome *b* hemes has been studied extensively using a variety of spectroscopic techniques, including electron paramagnetic (spin) resonance (EPR/ESR), magnetic circular dichroism (MCD), resonance Raman (RR), and low temperature absorption. These techniques have been applied to intact neutrophils, isolated granule populations, membrane suspensions, and detergent-solubilized, partially-purified flavocytochrome *b*. The information derived from these applications has provided insight into how the heme is ligated, and as a consequence, also provided indirect evidence regarding the domains of flavocytochrome *b* that might be involved.

ESR spectroscopic analyses on intact, resting porcine neutrophils at 4.2 K revealed *g* values of 2.85, 2.17, and 1.67 in a high magnetic field region, consistent with a low spin ferric heme. Assignment of the *g* = 2.85 value as *g_z* placed this parameter within the previously established values of 2.69-3.03, consistent with a bis-imidazole ligation. Thus, by inference, flavocytochrome *b* heme appeared to be coordinated in its fifth and sixth positions by histidine nitrogens (236). However, later experiments determined that porcine granulocyte suspensions isolated by these methods contained substantial eosinophil populations. A subcellular fraction derived from disrupted eosinophil membranes that lacked a flavocytochrome *b* reduced absorbance spectrum was shown to contain concentrated amounts of the cell-specific, eosinophil peroxidase (EPO), and a cyanide-sensitive, low-spin ESR signal at *g* = 2.87. Conversely, a separate fraction was isolated from *n*-heptyl- β -thioglucoside (heptylthioglucoside, HTG)-solubilized

neutrophil membranes that contained flavocytochrome *b* but was devoid of myeloperoxidase (MPO) or EPO. This fraction was able to produce superoxide in a cell-free assay and displayed an absorbance spectrum that was CO-insensitive. A cyanide insensitive, highly anisotropic, low-spin EPR signal with a g value of 3.2 ± 0.05 was also detected in this fraction, a value that was reportedly consistent with that of bis-histidyl-coordinated, *b*-type cytochromes. The authors thus concluded that flavocytochrome *b* was a hemoprotein that contained a hexa-coordinated low-spin heme component (237). Similar experiments conducted by the same group, but using highly concentrated, partially-purified flavocytochrome *b* from *n*-heptyl β -D-glucopyranoside (HTG) - solubilized porcine neutrophil membranes, again detected an ESR signal at $g = 3.26$ that was ascribed to flavocytochrome *b* (238,239). Additional EPR studies were conducted with the same model system that also incorporated near-infrared magnetic circular dichroism (NIR-MCD) and resonance Raman (RR) spectroscopy to examine the heme axial ligation (234). The data from these experiments concurred with their previous results supporting a bis-histidyl ligation scheme for the flavocytochrome *b* heme, and the spin state of the heme iron was shown to be sensitive to pH changes. Deviations from neutral pH resulted in the appearance of a high-spin ESR spectrum that was identical to that of hemoglobin suggesting that flavocytochrome *b* heme also utilized a histidine as its fifth or sixth axial ligand (240). A separate group was able to detect a similar low-spin EPR signal from purified, relipidated human flavocytochrome *b* with a g_z peak value of $g = 3.31$ (114). They considered this value to be too high for a simple bis-histidyl ligation,

and proposed that it resulted from histidine ligands that deviated from a parallel orientation, or possibly a non-histidine ligand.

The ligation of flavocytochrome *b* heme during the redox cycle has also been investigated to elucidate the mechanism of electron transfer to molecular oxygen during formation of superoxide. Fujii *et al.* used MCD spectroscopy to monitor the ratio of high to low-spin heme in flavocytochrome *b* purified from porcine neutrophils that resulted from changes in pH (240). Deviation from neutral pH resulted in a decrease in the low-spin heme signal concomitant with a reduction in superoxide production, suggesting that a low-spin heme state was essential for superoxide production. These observations were corroborated by the work of Isogai *et al.* (101) who investigated the thermodynamic and kinetic parameters of superoxide production under varied pH conditions using flavocytochrome *b* purified from porcine neutrophils. They concluded that the interactions between the internal axial ligands and the heme iron of flavocytochrome *b* were so strong that O₂ could not form an iron-O₂ intermediate. Therefore, the heme-O₂ interaction was proposed to take place near a solvent-exposed heme edge via an outer sphere mechanism while maintaining the heme in a low-spin, hexa-coordinated state.

A dose-dependent, reversible, inhibition of oxidase activity of either intact or lysed porcine neutrophils was achieved by addition of pyridine (239,241). The inhibition was correlated with changes in both the α - and Soret-band reduced absorbance maxima, as well as a shift in the ESR spectrum of flavocytochrome *b* from a low- to a high-spin state. The loss of the low-spin heme ESR signal was correlated with a decrease in oxidase activity, again suggesting that a low-spin heme iron was essential for superoxide

production. Investigation of the reaction rates between oxygen and reduced flavocytochrome *b* using rapid scanning and stopped flow spectrophotometry at low temperature (10 K) also failed to detect the formation of an oxygen intermediate. Furthermore, EPR measurements made under these same conditions revealed an invariant low spin heme signal suggesting that the heme iron remained in a low-spin, hexacoordinated state during the redox cycle (229). Lastly, resonance Raman spectra from both stimulated and resting human neutrophils, revealed only a low spin hexacoordinate heme iron (200).

In contrast, ESR data has been collected that indicated the presence of a high-spin heme iron during the redox cycle of flavocytochrome *b*, suggesting a transition from a hexacoordinated to a pentacoordinated state that would allow interactions between the heme and an extrinsic ligand, such as O₂. Cross-reacting, anti-P-450 reductase antibodies were used to immunoprecipitate flavocytochrome *b* from detergent solubilized membranes of either resting or stimulated porcine neutrophils (242). Cyanide insensitive, high-spin ESR signals at $g = 6.47$ and 5.49 were detected in the immunoprecipitate from only the stimulated cells, suggesting that the high-spin flavocytochrome *b* heme iron was associated with superoxide production.

Similarly, a low-spin signal at $g = 3.26$ and a high spin signal at $g = 6.0$ was detected in oxidized bovine flavocytochrome *b* (243). Addition of activating amounts of arachidonate resulted in a loss of the low-spin signal concurrent with an increase in the high-spin signal. Low temperature (77 K) absorption spectroscopy of detergent-solubilized flavocytochrome *b*, under the same conditions, revealed a 2.5 nm shift in the

Soret peak absorbance maximum following addition of SDS or arachidonate. These results were concurrent with an increased reactivity of the heme towards butyl isocyanide, with similar effects observed in activated, but not resting, intact neutrophils. Thus, the appearance of the transient high-spin ESR signal was attributed to a pentacoordinated heme iron and the consequent O_2^- production. Most recently, a separate group also showed that addition of arachidonate resulted in the appearance of a transient high-spin ferric iron that could bind cyanide, again suggesting that the high-spin heme contributed to O_2^- production (244).

Flavocytochrome *b* requires exceptionally careful handling due to the presence of high concentrations of endogenous proteases from the neutrophil cellular environment. A high spin heme iron was serendipitously detected in samples that had been kept at 10° C for several hours (200), and similarly, when intentionally kept at 40° C for 2 hours (245). Results from EPR and near-infrared magnetic circular dichroism (NIR-MCD) conducted on partially-purified, detergent-solubilized porcine-flavocytochrome *b* indicated the presence of a minor species with electronic characteristics that matched those of denatured bis-histidine *b*-type cytochromes. These spectra were shown to match those of bis-imidazole ferric protoporphyrin models, suggesting that the contributing species was denatured flavocytochrome *b* (234). Further evidence was provided by intentional heat denaturation or pH changes that resulted in the appearance of a high spin heme signal (240,244). These reports underscore the importance of sample handling, and suggest that the apparent inconsistencies in the data from different groups may be due to sample handling procedures. This proposal will remain speculative since EPR studies do not

routinely incorporate SDS-PAGE or other types of analyses that would reveal the integrity of the flavocytochrome *b* used in the analyses.

Subunit Location of the Hemes

There have been numerous studies directed at determining which of the flavocytochrome *b* subunits is responsible for heme coordination. Despite considerable effort put forth by several laboratories, the precise location of the hemes within the structure of flavocytochrome *b* remains controversial. Our most recent evidence clearly shows that there are two hemes per gp91^{phox}-p22^{phox} heterodimer. It is postulated that one heme resides solely within gp91^{phox}, and the other may be ligated by either gp91^{phox}, or p22^{phox}, or possibly shared between the subunits. In this section, the data that has contributed to the different biases is divided based on the subunit location of the hemes, and is presented in roughly chronological order.

Small Subunit Ligation. Early purification attempts of flavocytochrome *b* were generally directed at concentrating the heme component with no *a priori* knowledge of the protein's primary, secondary, tertiary, or quaternary structure. Also, knowledge of the high susceptibility of the flavocytochrome *b* to proteolysis was unknown, resulting in inconsistent reports of the size of the cytochrome, likely arising from different purification schemes and sample handling. Nonetheless, these inconsistencies, in

combination with the limited resources regarding structures of other hemoproteins, influenced preconceptions of the structure of flavocytochrome *b*.

Partial purification of a *b*-type cytochrome was achieved from Triton X-114-solubilized bovine neutrophil membranes using temperature-induced, biphasic partitioning, followed by several chromatography steps (227). The cytochrome bound CO, and had spectral characteristics and a reduction potential (-225 mV) similar to a previous report (87). SDS-PAGE analysis revealed three separate polypeptides with apparent masses of 11, 12, and 14 kDa, at least one of which was proposed to be flavocytochrome *b*. In a similar purification attempt, a 31.5 kDa polypeptide was found to co-purify with NADPH oxidase activity from detergent-solubilized, PMA-stimulated porcine neutrophil membranes (246). Analysis of the peptide revealed that it was phosphorylated when prepared from activated, but not from resting neutrophils. Based on this information, the authors concluded that flavocytochrome *b* was a 31.5 kDa polypeptide. The primary sequence determination of gp91^{phox} (20,95) revealed a protein that was in large part distinct from any known proteins, including other cytochromes. However, primary sequence determination of p22^{phox} (92) revealed a 31 amino acid residue stretch with 39% primary sequence identity to polypeptide I of cytochrome c oxidase (92). This observation, combined with the available purification data that suggested a smaller molecular mass species associated with heme, as well as other p22^{phox} attributes, such as the lack of glycosylation, suggested that p22^{phox} was the heme-bearing subunit of flavocytochrome *b*. Further evidence in support of this tenet was contributed by Nugent *et al.* who inferred a molecular mass of 22 kDa for the heme

bearing subunit based on sedimentation equilibrium studies of octyl glucoside (OG)-solubilized flavocytochrome *b*. The same group conducted additional experiments that used radiation inactivation of the heme spectrum and calculated the target size of the heme-bearing subunit to be 21 ± 5 kDa (247). In an attempt to purify the heme-bearing subunit of flavocytochrome *b*, Yamaguchi et al. (232) solubilized membranes prepared from frozen human neutrophils with OG and applied several chromatography steps. They were able to isolate a polypeptide with an apparent mass, by SDS-PAGE, of 20 kDa, that retained a small amount (3.9% of the starting amount) of both oxidized and reduced absorbance spectra identical to flavocytochrome *b* from previous reports. Although the amino acid composition was substantially different from that of previous reports (92) (142,226,227), the authors nonetheless concluded that $p22^{phox}$ was the sole heme-bearing subunit of flavocytochrome *b*. Lastly, CGD patients characterized by point mutations in $p22^{phox}$, with few exceptions (161,167), also resulted in loss of a detectable heme spectrum (159,248).

Heme Ligation Shared Between Subunits. There are several lines of indirect evidence that suggest that a heme may be shared between $gp91^{phox}$ and $p22^{phox}$. Initial predictions that $p22^{phox}$ was the sole heme-bearing subunit of flavocytochrome *b* were later challenged when it was determined that $p22^{phox}$ contained only a single invariant histidine (92,159,249). Since the spectroscopic data implied a bis-histidyl ligation of the heme, and the subunit stoichiometry was determined to be 1:1 (89-91), $p22^{phox}$ would only be able to provide a single histidine ligand. Therefore, if $p22^{phox}$ is involved with

heme coordination, the second histidine ligand would need to be provided by gp91^{phox} (90,236).

The subunits of flavocytochrome *b* are not disulfide linked, but are strongly associated and separable only under denaturing conditions that result in a loss of the heme absorbance spectrum (89). However, by separating the subunits using LDS-PAGE at 4° C, both subunits of flavocytochrome *b* were shown to electrophoretically migrate with heme (103). These results suggested that at least one heme could be associated with each of the subunits, though this conclusion was later questioned by Quinn and coworkers (230).

Genetic studies of CGD patients have shown that lesions in either subunit alone results in the absence of the other, suggesting they are mutually dependent for stable expression (17,19,26,160-162). Studies of Epstein-Barr virus (EBV)-transformed B-lymphocytes from p22^{phox}-deficient CGD patients indicated that the post-translational processing of gp91^{phox} appeared to be arrested at the stage of a 65 kDa, high-mannose precursor in the absence of p22^{phox} (210,250). This posit was confirmed when transgenic expression of p22^{phox}, in EBV-transformed B-lymphocytes derived from two autosomal CGD patients, restored full oxidase activity and complete processing of gp91^{phox} to its fully glycosylated form (250,251). Likewise, transgenic expression of gp91^{phox} in B-cells from three X-linked CGD patients (252), or a genetically altered gp91^{phox}-deficient PLB-985 human myeloid cell line (156), also resulted in stable flavocytochrome *b* heterodimer assembly and full restoration oxidase activity. Interestingly, recent work has revealed a link between heme incorporation and formation of a stable flavocytochrome *b*

heterodimer. Succinyl acetone (SA), a heme synthesis inhibitor (253), was added to PLB-985 myeloid cells induced by *N,N*-dimethylformamide (DMF) to undergo granulocytic differentiation (104). In this system, inhibition of heme synthesis resulted in a markedly reduced expression of mature $p22^{phox}$ – $gp91^{phox}$ heterodimers, but without affecting the expression levels of a 65-kDa high-mannose precursor of $gp91^{phox}$ (gp65). Addition of exogenous heme or removal of SA restored normal expression levels of the $gp91^{phox}$ – $p22^{phox}$ heterodimer and full oxidase activity.

Later studies, targeting the subcellular localization of gp65 during post-translational modification (254) in the $gp91^{phox}$ -deficient PLB-985 human myeloid cell line (156), suggested that gp65 and $p22^{phox}$ were assembled only after the gp65 carbohydrate was fully processed to the 91 kDa form of $gp91^{phox}$. Moreover, as shown previously (104), addition of SA during granulocytic differentiation had no effect on the expression of gp65, but inhibited the expression of the stable mature $gp91^{phox}$ - $p22^{phox}$ heterodimer. Most recently, studies that used tunicamycin to inhibit carbohydrate processing of gp65 in the same myeloid cell line revealed that addition of *N*-linked oligosaccharides to gp65 was not a necessary step for dimerization with $p22^{phox}$ (255). However, stable $gp91^{phox}$ - $p22^{phox}$ dimer formation was shown to require heme incorporation by gp65 prior to association with $p22^{phox}$, and failure to do so resulted in degradation of the individual monomers by the cytosolic proteasome. Conversely, in non-promyelocytic cell lines such as monkey kidney COS-7 cells and murine 3T3 fibroblasts, transgenic expression of fully processed $gp91^{phox}$ did not require the presence of $p22^{phox}$ although coexpression of both $p22^{phox}$ and $gp91^{phox}$ in these cells resulted in an

increased abundance of the mature gp91^{phox} glycoform (104). Further studies with this transgenic expression system determined that gp91^{phox} alone coordinated multiple hemes, while p22^{phox} was fully processed with no associated heme spectrum. However, the reduced Soret band absorbance spectrum from COS-7 gp91^{phox} alone was similar but not identical to either cells expressing both subunits, or native human neutrophil flavocytochrome *b*, and in the absence of co-expressed COS7 p22^{phox}, was unable to produce superoxide (230). Although there is no unambiguous evidence supporting a heme shared between gp91^{phox} and p22^{phox}, the experimental observations discussed above suggest that the possibility can not be dismissed.

Large Subunit Ligation. As protein data bases expanded, new structural motifs were discovered that allowed speculation about flavocytochrome *b* structure based on sequence and structural similarity. Fujii et al (239) proposed that the flavocytochrome *b* hemes of porcine neutrophils were coordinated solely by gp91^{phox} based on changes in the ESR spectrum following addition of pyridine. The resulting spectrum appeared similar to that of cytochrome *P450* from *Pseudomonas putida* suggesting a shift in the fifth axial ligand from a histidine to a vicinal thiolate. X-ray crystallographic studies of cytochrome *P450* from *P. putida* (256) revealed that the hemes were coordinated by a motif, FXXGXXXCLG, that was found to be conserved in cytochrome *P450* from several species. A similar seven residue spacing was likewise found between residues ⁷⁸FLRGSSAC⁸⁵ of gp91^{phox}, suggesting that this region might be involved in heme ligation of flavocytochrome *b* following exposure to pyridine. Thus, based on the

proximity of the heme-coordinating motif, in combination with previous ESR data that suggested bis-histidyl ligation, (237,238), and point mutation studies (162), the investigators proposed that His¹⁰¹ and His²⁰⁹ could provide the respective fifth and sixth heme ligands of the flavocytochrome *b* heme. Using a similar line of reasoning, but incorporating the results of additional spectroscopic studies (234), Fujii *et al.* again concluded that His¹⁰¹ and His²⁰⁹ of gp91^{phox} were the most probable heme-ligating residues, but did not rule out the possibility of a heme shared between gp91^{phox} and p22^{phox}. Another six-residue match derived from a heme-coordinating motif, TXXLAVHXXXV, originally found in the β -subunit of cytochrome *b*₅₅₉ of *Synechocystis* 6803 photosystem II (257), was also found in gp91^{phox} of human neutrophil flavocytochrome *b* in the region ²³²TAESLAVHHITY²⁴³, thus proposing another possible heme-ligating region.

Two non-promyelocytic cell lines, transgenic monkey kidney cells (COS-7) (230) and 3T3 fibroblasts (Dinauer, M.C., personal communication), that singly expressed either gp91^{phox} or p22^{phox}, or coexpressed both subunits of flavocytochrome *b*, were used to examine the heme coordination roles of the individual subunits. COS-7 cells that expressed only gp91^{phox} showed heme binding in the absence of COS-7 p22^{phox}, while cells that expressed only COS-7 p22^{phox} lacked a heme spectrum. Redox titrations of purified COS7 gp91^{phox} suggested two separate heme components with midpoint potentials of -233 and -264 mV, and coexpression with p22^{phox} had essentially no effect on these values. These data suggested that both hemes of flavocytochrome *b* were localized within gp91^{phox}. However, the reduced Soret band absorbance spectrum from

COS-7 gp91^{phox} alone was similar, but not identical to either cells expressing both subunits, or native human neutrophil flavocytochrome *b*. Moreover, in the absence of co-expressed COS-7 p22^{phox}, COS-7 gp91^{phox} was unable to produce superoxide.

A novel mutation in gp91^{phox} from an atypical X-linked CGD patient (258) resulted in a His¹⁰¹→Tyr transition and a complete loss of the heme absorbance spectrum even though immunoblotting of the neutrophil membranes revealed the presence of both flavocytochrome *b* subunits. A separate mutation in gp91^{phox} that resulted in a Arg⁵⁴→Ser mutation had no effect on flavocytochrome *b* expression levels, but resulted in the inability of the heme moiety to become reduced following activation in a cell-free assay. Furthermore, a change in the dithionite-reduced Soret absorbance spectrum compared to normal flavocytochrome *b* was observed, thus implicating the region in heme coordination (201).

Most recently, using a transgenic HEK-293 cell line, Maturana *et al.* expressed a mutant gp91^{phox} containing a His¹¹⁵→Leu substitution. The mutation abolished the characteristic α -band absorbance spectrum suggesting that His¹¹⁵ is involved with heme ligation (106). This proposal was corroborated by the mutational studies of Biberstine-Kinkade and colleagues (259) that suggested that His¹⁰¹, His¹¹⁵, His²⁰⁹, and His²²² are critical residues for gp91^{phox} heme ligation.

Heme Location Relative to the Membrane.

Inferences have been made of a stacked heme orientation within the membrane bilayer, coordinated by two transmembrane helices of gp91^{phox} based on similarities in primary sequence, spectral properties, and redox potentials between flavocytochrome *b* and select members of the ferredoxin-NADP⁺ reductase family, including FRE1 ferric reductase of *Saccharomyces cerevisiae* (94,196,197,235).

Thermodynamic constraints (99) and the intrinsic hydrophobic nature of the heme would suggest placement within either a hydrophobic protein domain, or within the NH₂-terminal transmembrane spanning regions. Indirect evidence supporting this tenet was provided by the studies of Fujii *et al.* (260) that were intended to determine the site of oxygen reduction. Doxylstearic acids (DS) that were spin-labeled at the 5,7,12, or 16 positions of the stearic acid were separately incorporated into intact porcine neutrophils. Following stimulation by phorbol myristate acetate (PMA), the resulting O₂⁻ reduced the nitroxide free-radical and quenched the ESR signal of the 5-DS. The 5-DS reduction was inhibitable by copper salicylate, a hydrophobic O₂⁻ scavenger, but not by superoxide dismutase suggesting that the site of O₂⁻ release is located within a hydrophobic environment approximately 4-5 Å from the plasma membrane surface. However, interpretation of these results to include prediction of heme location would require the assumption that a heme is the terminal electron acceptor prior to reduction of molecular oxygen, a hypothesis that has not been proven.

To date, the only direct experimental evidence supporting heme placement within the transmembrane regions of flavocytochrome *b* has been acquired by our laboratory. Digestion experiments by Quinn et. al. were conducted to resolve conflicting reports regarding the number of hemes and the flavocytochrome *b* subunit responsible for their coordination. The proteases *Staphylococcal* V8 or trypsin were used on either purified or neutrophil plasma membrane bound flavocytochrome *b*. Proteolysis of purified flavocytochrome *b* with V8 for periods of up to 240 minutes completely degraded both subunits and resulted in a 20% loss of absorbance without otherwise affecting the flavocytochrome *b* absorbance spectrum. Also, exposure of neutrophil membranes to either V8 or trypsin for 4 hours, followed by centrifugation, showed the heme to be associated with the membrane pellet, suggesting that the hemes reside within the lipid bilayer.

Thesis Statement.

The studies discussed above suggest that the hemes of flavocytochrome *b* are coordinated by the NH₂-terminal regions of the protein. These regions are primarily hydrophobic in nature and contain many stretches that are predicted to be membrane-spanning α -helical structures. We intended to acquire direct experimental evidence regarding the location of the flavocytochrome *b* hemes by using controlled proteolysis in combination with HPLC size-exclusion chromatography. The details of these experiments, presented in Chapter 2 of this dissertation, indicate that the flavocytochrome

b hemes are contained within the NH₂-terminal regions of the protein. This observation suggested that heme-mediated, resonance energy transfer (RET) fluorescence quenching experiments could be used to investigate the molecular geometry of flavocytochrome *b*. As discussed above, there are several interactions that are likely to occur between the cytosolic subunits of the oxidase and flavocytochrome *b*. Although the precise details of these interactions remain to be determined, the final unanswered question is, how do these interactions confer activity to the oxidase? We propose that the final step in oxidase activation involves a transition of flavocytochrome *b* to an active state conformer. To test this hypothesis, we designed a system of extrinsic fluorescent probes that allowed detection of minute structural perturbations in both membrane-bound and partially-purified, detergent solubilized, flavocytochrome *b*. These probes were then used to demonstrate that changes in flavocytochrome *b* structure occur during exposure to compounds known to activate the oxidase *in vitro*. The details of these experiments are presented in Chapters 3 and 4 of this dissertation.

Experimental Procedures.

Proteolysis.

Proteolysis experiments were conducted using *Staphylococcus* V8 protease (Endoproteinase Glu-C), which shows a high degree of specificity for the C-terminal side of glutamic acid residues, and a roughly 3000-fold lower specificity for aspartic acid

residues (261). The protease was utilized under highly controlled conditions as described in Chapter 2 of this dissertation. Given the substantially lower selectivity of the protease for aspartate residues, the protease is expected to target the glutamic acid residues (32 in gp91^{phox} and 12 in p22^{phox}) rather than the aspartic acid residues (16 in gp91^{phox} and one in p22^{phox}). These experiments are a continuation of those conducted previously by Quinn *et al.* in our laboratory (103). The experimental details of these experiments are provided in Chapter 2 of this dissertation.

Resonance Energy Transfer.

Genome sequencing information suggests that roughly 30% of the predicted proteins are integral membrane proteins. High resolution structures are currently obtained by using X-ray or electron diffraction crystallography (for reviews see refs (262))(263), and solution or solid state nuclear magnetic resonance spectroscopy (NMR) (264). However, application of diffraction methods to the majority of transmembrane proteins has been hindered by the failure of these molecules to form usable crystals, and NMR spectroscopy is limited to proteins with masses less than ~ 18-20 kDa. Currently, less than 20 high resolution structures of membrane proteins exist. Since structural details of proteins are essential to understanding and predicting function, lower resolution methods have been developed to provide information about proteins for which no high resolution information is currently attainable.

One such method was proposed by Th. Förster who established the theoretical relationship between a pair of spectrally matched chromophores, their average distance of separation, and the rate of energy transfer between them (265). The theory states that electronic excitation energy from a fluorescent donor molecule can be transferred to a spectrally-matched acceptor, and the rate or probability of this occurrence is proportional to the inverse sixth power of the distance separating the two molecules. Later experimental work confirmed that the theory was correct and led to the development of the technique as a "spectroscopic ruler" (266,267), thus providing a method for measurement of distances between donors and acceptors over a range of $\sim 10 - 100 \text{ \AA}$. This type of spectroscopy is named fluorescence resonance energy transfer (FRET) or more accurately, resonance energy transfer (RET), emphasizing that the energy transfer is a non-radiative process (268).

Cascade Blue Fluorescent Dye. Our RET systems utilize the acetyl azide derivative of the trisodium salt of 1,3-Trisulfo-8-pyrenyloxyacetylhydrazide, or Cascade Blue[®] acetyl azide (CCB) (269) as the extrinsic fluorescence donor moiety, and the endogenous hemes of flavocytochrome *b* as the acceptors. This derivative of CCB is amine reactive thus allowing labeling of proteins such as wheat germ agglutinin (WGA) or monoclonal antibodies (mAbs) specific for flavocytochrome *b*. With this system, changes in the spatial relationships between the CCB donors and heme acceptors affects the quantum yield of the CCB moieties. We can therefore detect minute changes in the

geometry of flavocytochrome *b* by monitoring the steady-state fluorescence of the CCB donor.

RET Distance Calculations. Fluorescence quenching events and any subsequent relaxation can be interpreted as a changes in the spatial relationships between the donors and acceptors as defined by Förster. The average distance between the donor and acceptor pairs are calculated as follows; The spectral overlap integral (*J*) of the donor fluorescence and acceptor absorption is defined by Equation 1 (265,268).

$$J = \int F_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda / \int F_D(\lambda) d\lambda \quad (\text{Eq. 1})$$

$F_D(\lambda)$ is the measured fluorescence intensity (in counts/sec) of the CCB donor at wavelength λ (in nm), and $\epsilon_A(\lambda)$ is the molar extinction coefficient of the flavocytochrome *b* heme acceptor at the same wavelength in units of $(\text{cm M})^{-1}$. Using the indicated units, *J* is in units of $(\text{cm M})^{-1}(\text{nm})^4$. Since the values for λ are not a continuum, but discrete steps determined by the monochromator settings, $d\lambda$ can be substituted with $\Delta\lambda$, and the calculation can be done as a simple summation (Equation 2)(270) using a spreadsheet format, with fluorescence spectra corrected for the wavelength-dependent instrument response using the software functions provided by the manufacturer (Felix software ver 1.1, 1996 PTI, Inc).

$$J = \sum_i F_D(\lambda_i) \epsilon_A(\lambda_i) \lambda_i^4 / \sum_i F_D(\lambda_i) \quad (\text{Eq. 2})$$

R_0 is the Förster distance, an internal reference for distance calculations derived from the intrinsic spectroscopic properties of the donor and acceptor molecules as defined in Equation 3. Förster first defined this parameter as the distance separating the donor-acceptor pair at which 50 percent of the excitation energy of the donor is nonradiative, or quenched (265).

$$R_0 = 0.211 (JK^2 n^{-4} \Phi_D) \quad (\text{Eq. 3})$$

K^2 is the relative orientation factor between the donor and acceptor dipole moments. Since the relative dipole orientation between the CCB and heme moieties of the conjugate probes is unknown, we have chosen $K^2 = 2/3$, the average value over all orientations. The intrinsic planar symmetry of the heme molecule relative to the donor dipole imposes at most a 20% error in the upper limit of accuracy of any distance approximations (271). n is the refractive index of the medium separating the donor and acceptor, chosen to be 1.44. Φ_D is the quantum yield of the CCB-WGA molecule and was determined by the method outlined in Parker and Rees (272), using Equation 4.

$$F(\lambda) = I(\lambda) [1 - 10^{-\epsilon(\lambda)cl}] \Phi_D \quad (\text{Eq. 4})$$

$F(\lambda)$ is the measured fluorescence intensity of the CCB conjugate in quanta/unit time at wavelength λ . $I(\lambda)$ is the intensity of the exciting light, or the instrument response function, in the same units, c is the concentration of the fluorescing solute, determined by absorbance, l is the optical path length in cm, and $\epsilon(\lambda)$ is the molar extinction coefficient of the fluorescing probe at wavelength λ . As a reference standard to determine $I(\lambda)$, quinine sulfate in 0.1N H_2SO_4 , with $\epsilon_{346} = 1.09 \times 10^4$ and $\Phi_D = 0.51 \pm 0.03$ if $A_{346} < 0.02$ AU ($< 1 \mu M$) (273) was used. Determination of $I(\lambda)$ allowed calculation of the quantum yield (Φ_D) for the CCB-conjugate by Equation 5.

$$I(\lambda)_{\text{quinine sulfate}} = F(\lambda)[(\Phi_D)(1-10^{-\epsilon(\lambda)cl})]^{-1} \quad \text{for } \lambda = 346 \text{ nm} \quad (\text{Eq. 5})$$

Evaluation of the instrument response function $I(\lambda)$, at $\lambda_{\text{ex}} = 376$ nm with a quinine sulfate standard at the same optical density at 376 nm, normalized with respect to $\lambda_{\text{ex}} = 346$ nm, yields the corrected instrument response at 376 nm, the excitation wavelength of CCB. The corrected instrument response at 376 nm, $I_{\text{corr}}(376 \text{ nm}) = I(346 \text{ nm})(\alpha)$, where α is determined from Equation 6:

$$\alpha = 1 - \{[I(376 \text{ nm}) - I(346 \text{ nm})] / I(346 \text{ nm})\} \quad (\text{Eq. 6})$$

This value allows determination of the quantum efficiency of the donor probe by Equation 7.

$$\Phi_{D,CCB-WGA} = F(\lambda)I_{\text{corr}}(\lambda)[1-10^{-\epsilon(\lambda)cl}]^{-1} \text{ for } \lambda_{\text{ex}} = 376 \text{ nm} \quad (\text{Eq. 7})$$

With units of λ in nm, and $\epsilon_A(\lambda)$ in $(\text{cm M})^{-1}$, R_0 is in units of Å (268). Measurement of the relative steady-state fluorescence intensity of the donor probe in the presence (I_{DA}), or absence (I_D), of the flavocytochrome *b* acceptor molecule allows calculation of the resonance energy transfer efficiency (E) between the donor - acceptor pair by the relationship,

$$E = 1 - (I_{DA}/I_D) \quad (\text{Eq. 8})$$

From this value, the average radial distance (r_{av}) separating the donor - acceptor pair can be calculated from equation 9.

$$r_{\text{av}} = R_0(E^{-1}-1)^{1/6} \quad (\text{Eq. 9})$$

Wheat Germ Agglutinin. Wheat germ agglutinin (WGA) is a plant lectin purified, in this case, from *Triticum vulgaris*. It is a member of a highly conserved family of chitin binding proteins, *Gramineae* that are grouped based on the presence of a conserved disulfide-rich repeat (274). WGA is heterodimeric, comprising two identical molecular mass subunits, or "protomers", each 17.5 ± 1 kDa, designated I

and II. Each protomer is composed of four, 41 amino acid repeats, or "domains", named A_I, B_I, C_I, D_I, with the subscript referring to the domain's respective subunit (275). WGA exhibits a high degree of specificity and affinity for two types of *N*-acetylated sugars, *N*-acetyl-D-glucosamine (GlcNAc, NAG) and to a lesser extent, *N*-acetyl-D-neuraminic acid (NeuNAc). Extensive effort has been directed towards elucidation of the saccharide binding modes of this molecule using a variety of techniques, the most effective being X-ray diffraction crystallography augmented with computer modeling (see (276) and the references therein). Given the appropriate conditions, native WGA will spontaneously form crystals. However, introduction of any of the known ligands at concentrations as low as 10 μ M rapidly dissolves the crystals thus thwarting any attempts at cocrystallization. Determination of the saccharide binding modes was accomplished by first forming native WGA crystals, crosslinking them with gluteraldehyde, and then exposing them to mM concentrations of ligand over a period of several days. This technique allowed identification four domains within each monomer that were established as carbohydrate recognition domains (CRD) based on ligand occupancy (275). Two of the four domains were found to have higher occupancy leading to the designation of *primary* for the two dominant domains and *secondary* for the presumed lower affinity sites, though site occupancy may not be a direct reflection of binding strength due to crystal packing artifacts that interfere with site accessibility (276). Only two of the four sites per monomer have been detected in NMR solution studies suggesting that the interactions with the secondary sites are too weak to be detectable in solution (277-280). For the

studies detailed in chapters 3 and 4 of this dissertation, the ability of WGA to bind NAG and its multimers was exploited as a means to position a CCB-conjugated WGA (CCB-WGA) within resonance energy transfer distance of the endogenous flavocytochrome *b* hemes.

Monoclonal Antibodies. Tables 1.1 and 1.2 show the large number of α -flavocytochrome *b* that are currently available. For the experiments discussed in Chapter 4 of this dissertation, α -flavocytochrome *b* mAbs 44.1 and 7D5, and a control mAb H7 specific to hCAP-18 (281-283) were chosen. As discussed in the text of the Anti-flavocytochrome *b* Antibodies section, the epitopes for mAbs 44.1 and 7D5 have been fully characterized.

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CHAPTER 2

IDENTIFICATION OF A SPECTRALLY STABLE PROTEOLYTIC FRAGMENT OF HUMAN NEUTROPHIL FLAVOCYTOCHROME *b* COMPOSED OF THE NH₂-TERMINAL REGIONS OF gp91^{phox} and p22^{phox}Introduction

The NADPH oxidase of human neutrophils is a multi-subunit, membrane-associated complex that is crucial for host protection against invading pathogens (1-7). The redox center and the only membrane-spanning component of the oxidase is flavocytochrome *b*₅₅₈, a heterodimeric protein comprised of an extensively glycosylated, 91 kDa large subunit (570 amino acid residues), gp91^{phox}, and a 22 kDa non-glycosylated small subunit (192 amino acid residues), p22^{phox} (8,9). Intracellular binding sites for both FAD and NADPH have been identified on gp91^{phox} (10-14). Flavocytochrome *b* functions as the terminal electron carrier prior to reduction of extracellular molecular oxygen to the antimicrobial precursor, superoxide anion (O₂⁻) (2,15-18). Flavocytochrome *b* has also been proposed to function as a voltage-gated proton transporter that maintains intracellular pH and membrane potential during the oxidative burst (19,20).

The characteristic absorbance spectrum of flavocytochrome *b* is attributed to the presence of heme prosthetic groups that are non-covalently coordinated by histidine residues within the protein. Due to the tenuous nature of this ligation scheme, the heme spectrum is lost under conditions that separate the individual subunits (21), though heme

remains associated with both subunits during electrophoresis at low temperature with lithium dodecyl sulfate (LDS)-PAGE (22). Additionally, the detergent-solubilized protein is highly susceptible to proteolysis by the numerous endogenous phagocyte proteinases. These and other factors have prevented direct identification of the regions of the flavocytochrome *b* heterodimer that are responsible for heme coordination. There are, however, several lines of indirect evidence deriving from mutational (23,24) and spectroscopic analyses (25,26) that implicate regions of gp91^{phox} with heme binding.

Inferences have also been made of a stacked heme orientation within the membrane bilayer, coordinated by two transmembrane helices of gp91^{phox} based on similarities in primary sequence, spectral properties, and redox potentials between flavocytochrome *b* and select members of the ferredoxin-NADP⁺ reductase family, including FRE1 ferric reductase of *Saccharomyces cerevisiae* (27-30). Likewise, heme localization within gp91^{phox} has been inferred based on similarities to heme-coordinating regions of cytochrome P450 of *Pseudomonas putida* (31), and the β -subunit of cytochrome *b*₅₅₉ of *Synechocystis* 6803 photosystem II (32). These arguments and the intrinsic hydrophobic nature of the heme molecule would suggest placement within the NH₂-terminal, putative membrane-spanning regions of flavocytochrome *b* (33-35) although there is no direct evidence to support this assumption.

The intent of this study was to isolate and identify proteinase-stable heme-ligating regions of flavocytochrome *b*. Partially-purified flavocytochrome *b* was exposed to *Staphylococcal* V8 proteinase (Endoproteinase Glu-C). HPLC size exclusion chromatography was then used to isolate proteolytic fragments that retained the

characteristic 414 nm heme absorbance spectrum. With this approach, we successfully isolated a spectrally-native polypeptide core that was then characterized by amino acid sequencing and immunoblotting. The fragment was found to be a heterodimer comprised of the NH₂-terminal 336 to 363 amino acid residues of gp91^{phox}, and the NH₂-terminal 169 to 171 amino acid residues of p22^{phox}. The core fragment retained 74% of the native heme absorbance, suggesting that it contained all of the heme-ligating regions of flavocytochrome *b*. These findings suggest that the hemes are positioned intra- or juxtamembrane within the NH₂-terminal predicted transmembrane-spanning regions of the protein.

Abbreviations

The abbreviations used are: AU, absorbance units; CGD, chronic granulomatous disease; CMC, critical micelle concentration; DAD, diode array detector; DDM, dodecylmaltoside; DTT, dithiothreitol; Flavocytochrome *b*; cytochrome *b*₅₅₈, cytochrome *b*₅₅₉; Glu-C, glutamic acid, carboxyl terminal side; HPLC, high performance liquid chromatography; mAb, monoclonal antibody; λ_{max} , absorbance maximum; NMWCO, nominal molecular weight cutoff; mAb, monoclonal antibody; O₂⁻, superoxide anion; OG, octylglucoside, octyl- β -glucopyranoside; *phox*, phagocyte oxidase; SDS, sodium dodecyl sulfate; TMBZ, 3,3',5,5'-Tetramethylbenzidine UV, ultra violet; V8 proteinase, *Staphylococcal* V8 endoproteinase Glu-C.

Experimental Procedures

Materials.

KCl, NaCl, EDTA, NaN_3 , silver nitrate, sodium carbonate, gelatin, Trizma Base (Tris), [ethylenebis(oxyethylenenitrilo)]tetra acetic acid (EGTA), and syringe filters (Whatman, 25 mm diameter, polyethersulfone membrane, 0.2 μm pore size) were purchased from Fisher Scientific (Tustin, CA). N-acetylglucosamine (GlcNAc), N-N'-diacetylglucosamine (chitobiose), Heparin-Sepharose[®] 4B beads, N-formyl-Met-Leu-Phe, dihydrocytochalasin B, Na_2 ATP, chymostatin, wheat germ agglutinin (WGA), diisopropylfluorophosphate (DFP), sodium dithionite, bovine serum albumin (BSA), HANKS' balanced salts, MgCl_2 , NaH_2PO_4 , TRIZMA-HCl (Tris-HCL), hemin chloride (bovine), hydrogen peroxide 3% (w/w) solution, lithium dodecyl sulfate (LDS), pyridine (HPLC grade), glutathione, free-acid, reduced form, thioglycolic acid (thioglycollate, mercaptoacetic acid), free-acid, trifluoromethanesulfonic acid (TFMS), iodoacetamide, 3,3',5,5'-Tetramethylbenzidine (TMBZ)(free base), and Proteinase Inhibitor Cocktail (P8340) were from Sigma Chemical Co. (St. Louis, MO). Polyvinylidene difluoride (PVDF) membrane (0.2 μm pore size) was from Bio-Rad Laboratories (Richmond, CA). Triton X-100 detergent was from EM Sciences Co. or Sigma Chemical. [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] (HEPES), Gammabind[®], and CNBr-activated Sepharose 4B beads were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). Ultrapure SDS was purchased from United States Biochemical

(Cleveland, OH). N-octyl- β -D glucopyranoside (OG, octylglucoside), dithiothreitol (DTT, Cleland's reagent), and phenylmethylsulfonyl fluoride (PMSF), were from Calbiochem-Novabiochem Corp (La Jolla, CA). The BCA protein assay and BlueRanger® prestained molecular weight markers for SDS-PAGE were from Pierce, Inc. (Iselin, NJ). Other prestained molecular weight markers were supplied by GIBCOBRL Life Technologies (Carlsbad, CA). Endoproteinase Glu-C (Proteinase V8 salt free, lyophilisate, sequencing grade) from *Staphylococcus aureus* V8 was from Boehringer Mannheim (Mannheim, FRG). NBT-BCIP alkaline phosphatase developer kit for immunoblots was from Kirkegaard and Perry Laboratories (KPL) (Gaithersburg, MD)

Buffers.

The following buffers were used in this work: Heparin wash buffer: 50 mM NaH_2PO_4 , 1 mM EGTA, 1 mM MgCl_2 , 0.1% (v/v) Triton X-100, 2 mM NaN_3 , pH 7.4, supplemented with 0.1 mM DTT, 10 $\mu\text{g/ml}$ chymostatin, 0.2 mM PMSF (final concentrations) just prior to use; Heparin elution buffer: either 75 mM or 2.0 M NaCl, 50 mM NaH_2PO_4 , 2 mM NaN_3 , pH 7.4, 1 mM EGTA, 1 mM MgCl_2 , 0.1% (v/v) Triton X-100, supplemented with 0.1 mM DTT, 10 $\mu\text{g/ml}$ chymostatin, 0.2 mM PMSF (final concentrations) immediately prior to use; HPLC column buffer: 150 mM NaCl, 50 mM NaH_2PO_4 , 1 mM EGTA, 1 mM MgCl_2 , 0.1% (v/v) Triton X-100, 2 mM NaN_3 , 0.1 mM DTT, pH 7.4. TS Buffer; 200 mM Tris Base, 2% SDS, pH 8.0.

Partial Purification of Flavocytochrome *b*

Isolation of human PMNs from whole blood and purification of flavocytochrome *b* was carried out as described previously (9,36) or with the following modifications: the wheat germ agglutinin affinity steps were eliminated to reduce the degree of sample handling, thus resulting in higher recoveries of flavocytochrome *b* without having an apparent effect on the overall purity. After loading, the heparin column containing the flavocytochrome *b* was washed with 10-20 bed volumes of heparin wash buffer, and eluted using either a 50 mM to 2.0 M NaCl gradient, or a 6 mL bolus of 1.0 M NaCl, both in heparin elution buffer. 1 mL fractions were collected and the peak flavocytochrome *b*-containing fractions were pooled and concentrated to a final volume of ~ 1 mL using a 30 kDa NMWCO centrifugal concentration device. As a final purification step prior to digestion, the retentate was subjected to an HPLC size exclusion chromatography step, as described below, and collected in 400 μ L aliquots. Aliquots that were reserved for predigest controls had PMSF and chymostatin added to 1 mM and 10 μ g/mL, respectively. Beginning with the solubilization step, all purification and digestion steps were accomplished in 1 day with all samples kept on wet ice prior to digestion. Flavocytochrome *b* heme content prior to heparin purification was quantitated using the reduced *minus* oxidized spectrum at 558 nm using $\Delta\epsilon_{558} = 29.3 \text{ (mM cm)}^{-1}$ (37), blanked against control buffer, and reduced by addition of freshly mixed sodium dithionite in deionized water to a final concentration of 10 mM. After heparin purification, flavocytochrome *b* heme quantitation was carried out using $\epsilon_{414} = 130.8 \text{ (mM cm)}^{-1}$ (37)

blanked against buffer. Absorbance values were determined using either a Hewlett Packard HP 8452A diode-array UV-Visible spectrophotometer or a Molecular Dynamics Spectra-Max 250, environmentally controlled, 96 well micro titer plate, UV-Visible spectrophotometer. When necessary, the samples were sonicated using either a Fisher 50 probe style Sonic Dismembrator, model XL2005, or a Fisher- brand bath sonicator, model FS30.

HPLC Size Exclusion Chromatography of Flavocytochrome *b*.

All HPLC analyses were carried out using a Hitachi, LS-6200 HPLC with an F-1050 fluorescence detector connected in series with an L-7450A UV-Visible Diode Array Detector. Size exclusion chromatography was performed using a Pharmacia Superdex 200 HR[®], 10-30 column, maintained at 4° C with a flow rate of 0.4 mL/min, equilibrated for a minimum of 1.5 h prior to sample injection. The column was calibrated using size exclusion chromatography standards (Bio-Rad # 1511901) that included thyroglobulin, 670 kDa; gammaglobulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa, and vitamin B-12, 1.35 kDa. The standard curve was fitted using a non-linear regression algorithm (GraphPad Prism version 3.01 for Windows 95, GraphPad Software, San Diego California USA, www.graphpad.com, Copyright (c) 1994-1999 by GraphPad Software). Prior to HPLC size exclusion chromatography, all samples were bath sonicated for 1-2 sec and passed through a 0.2 µm pore size syringe filter. HPLC elution fractions were collected from the column in 400 µL aliquots at 1 min intervals. Time point samplings

for V8 digestions were removed from the digestion vessel and placed on wet ice with proteinase inhibitors prior to separation by HPLC size exclusion using the same procedures as for intact flavocytochrome *b*. All absorbance values were corrected for dilution and differences in HPLC injection volumes.

V8 Digestion of Flavocytochrome *b*.

Lyophilized V8 proteinase was reconstituted at 50-500 $\mu\text{g/mL}$ in either distilled water or HPLC column buffer and added to the partially-purified flavocytochrome *b* samples, while mixing, to a final ratio of 1:15 (w/w), proteinase to flavocytochrome *b* heme (assuming a flavocytochrome *b* heterodimer molecular mass of 110 kDa). The digestion was carried out at 37° C in a continuously-stirred vessel, and terminated by placement on wet ice and addition of PMSF and chymostatin to final concentrations of 1mM, and 10 $\mu\text{g/mL}$, respectively. Digested samples were stored on ice at 4° C until further analyses were conducted. Initial quantities of purified flavocytochrome *b* used for digestion ranged between 1.8 - 2.7 nmoles heme. The fractions retained from the HPLC runs had additional PMSF and chymostatin added to the same concentrations as above, and were kept on ice until further analyses were conducted.

Modified TMBZ Heme Quantitation Assay.

Heme quantitation of HPLC fractions was carried out by modifying the procedure originally described (38,39) to include addition of ethanol to the assay mixture to a nominal concentration of 50% (v/v). Assays were conducted in 96 well micro titer plates and absorbance values were measured with a Molecular Dynamics Spectra-Max 250 UV-Visible, environmentally controlled micro titer plate spectrophotometer. Reagents used in the assay were prepared immediately prior to use. The short incubation times combined with the large number of samples that were simultaneously tested, required the use of a multi-tipped pipetter to reduce timing errors in the mixing of the individual micro titer plate wells. Hemin standard solutions were prepared for initial quantitation as reduced, alkaline pyridine-solubilized hemochrome (37) by addition of 0.5 N NaOH to a final sample concentration of 0.075 N, and 4.0 M pyridine to 2.1 M final concentration. The standards were then reduced by addition of sodium dithionite to a final concentration of 10 mM and quantitated using the reduced - oxidized molar extinction coefficient, $\Delta\epsilon_{556-540} = 20.7 \text{ (mM cm)}^{-1}$ (37). TMBZ solution consisted of 10 mg of dry TMBZ mixed with 500 μL of glacial acetic acid, filtered through a 0.2 μm pore size polyethersulfone membrane filter. Per well, 15 μL of the TMBZ solution was mixed with 30 μL of hemochrome sample, 100 μL of absolute ethanol, and incubated at ambient temperature for 2 min. At the end of the two min, 15 μL aliquots of 3% H_2O_2 were added per well and the mixtures incubated at ambient temperature for 5 min. The absorbance was then measured at 660 nm and the hemin concentration of the samples determined relative to

the standards. All absorbance values were corrected for dilution and differences in HPLC injection volumes.

Immunoblot Analyses and Silver Staining.

Flavocytochrome *b* containing fractions were resolved by SDS-PAGE using 5-20% acrylamide gradient gels (40), and electrophoretic transfer of protein to nitrocellulose for immunoblotting was performed as described (41). Anti-gp91^{phox} primary antibodies used were mouse mAbs (NL10, CL5, NL7)⁴, and 54.1 (42,43), and anti-peptide rabbit polyclonal antibodies KIS (Baniulus, D., unpublished), and KQS (44). Anti-p22^{phox} primary antibodies used were mouse mAbs (NS1, NS2, NS5, CS9)(J. B. Burritt, unpublished), and 44.1 (42,43), and the rabbit polyclonal antibodies, anti-peptide EAR (45), and R3179 (9) produced by injecting rabbits with intact flavocytochrome *b*. Nitrocellulose transfers probed with primary antibodies were then probed with goat-anti-rabbit, or goat anti-mouse, alkaline phosphatase-conjugated secondary antibodies, and visualized using the KPL NBT-BCIP developer kit. Silver staining of polyacrylamide gels was carried out by overnight rocking in 50% methanol, 12% acetic acid in water, followed by 3, 20 min washes in 50% ethanol-water. Gels were then drained, completely submerged for 1 min in a solution containing 0.2 g/L sodium thiosulfate, and washed 3 times, 30 sec each with distilled water. After draining, 100 mL of a solution containing 0.2 g/L silver nitrate, 1.0 mL/L formaldehyde, in water was added and the gels were rocked for 20-30 min. The gels were then rinsed 3 times with water, 30 sec each, and

developed by addition of 200 mL of 60 g/L sodium carbonate, 1.0 mL/L formaldehyde, and 30 mL/L of the 0.2 g/L sodium thiosulfate in distilled water, and stopped by addition of 25% isopropanol and 10% acetic acid in water.

Preparation of Flavocytochrome *b* for Amino Acid Sequence Analysis.

NH₂- terminal sequence analyses by Edman degradation were performed by Harvard Microchem (Cambridge, MA), and the samples prepared as per their recommendations. To reduce the quantity of ubiquitous keratin in the SDS-PAGE reagents, twice recrystallized SDS (46) and distilled water that had been filtered through a 10 kDa NMWCO filter were used. All other aqueous reagents were filtered through a 0.2 μ m pore size membrane filter.

Inadvertent chemical modification of the NH₂-termini of peptides to be sequenced was avoided by including the following modifications to the SDS-PAGE protocol (40). 5-20% gradient polyacrylamide resolving gels were allowed to polymerize overnight at ambient temperature. The stacking gel was poured, allowed to polymerize, then pre run without sample for 45 min at 4 mA constant current in the presence of Running Buffer containing 5 μ M reduced glutathione. The glutathione-containing buffer was removed, the samples loaded, and the electrophoresis conducted at 40 mA constant current using new Running Buffer that contained 100 μ M thioglycolic acid (47). Following SDS-PAGE, the proteins were transferred to PVDF membrane, stained with amido black for visualization (46), excised from the membrane, and the strips washed 3X by gentle

vortexing, 15 sec each, in filtered distilled water. The individual strips were then air-dried and placed in sealed plastic tubes for shipment. The diffuse band centered at ~ 90 kDa and the consolidated band at 22 kDa were excised for NH₂-terminal sequence analyses of the nondigested flavocytochrome *b*. The single band at 17 kDa and the entire broad band from ~ 50 to 70 kDa were excised from the PVDF membrane for NH₂-terminal sequence analyses of the 1 h digest fragments.

Reduction and Alkylation.

Samples were reduced by addition of an equal volume of DTT solution (50 mM DTT in TS Buffer) and heated 4-5 min at 90° C. Alkylation was performed by adding 1/10 volume (sample + DTT mixture) of iodoacetamide stock (46 mg of iodoacetamide dissolved in 200 mM Tris base, pH 8.0), followed by incubation at 90° C for 3-4 min with IgG used as a procedural control. Iodoacetamide was added without DTT treatment to sample controls. All samples were then added to sample loading buffer, separated by SDS-PAGE and immunoblotted as described above.

Deglycosylation of Flavocytochrome *b*.

For chemical deglycosylation, both intact and digested polypeptides were precipitated by addition of 80% (v/v) trichloroacetic acid (TCA) in distilled water to a final sample concentration of 15% (v/v). Samples were then cooled at -20° C for 30 min,

followed by centrifugation at $180,000 \times g$ for 15 min. The supernatant fraction was removed, and the pellet was then washed twice in -20°C acetone to remove the TCA, and either allowed to air dry or purged under a stream of dry argon at room temperature. The pellet was then resuspended into $100 \mu\text{L}$ of neat trifluoromethanesulfonic acid (TFMS) (48,49) by bath sonication in sealed tubes, purged with argon, and incubated on ice for 3 h in a ventilation hood. At the end of the incubation, the samples were cooled to less than -20°C by immersion in a mixture of dry ice and ethanol. Neat pyridine, likewise cooled to less than -20°C , was then slowly added to terminate the reaction. The volatile organic phase was removed under a stream of dry argon at room temperature, and the resulting gel resuspended in distilled water, and dialyzed against several changes of 10 mM PBS at 4°C . The protein was precipitated by addition of TCA to 15% (v/v), incubated at -20°C for 15 min, and pelleted by centrifugation at $20,000 \times g$ for 15 min at 4°C . The resulting pellets were washed twice with -20°C neat acetone, and pelleted each time by centrifugation at $20,000 \times g$, for 10 min at 4°C . Samples were then reduced and alkylated as described above, mixed with loading buffer, and added directly to the lanes for separation by SDS-PAGE. Enzymatic deglycosylation of flavocytochrome *b* was carried out using PNGase F. HPLC size exclusion chromatography fractions were denatured by addition of SDS to 0.5% in the presence of 10 mM DTT and $1 \mu\text{L/mL}$ proteinase inhibitor cocktail, and then heated to 100°C for 10 min. Reagents supplied by the manufacturer were then added as per their instructions and incubated at 37°C for 1 h with intermittent mixing.

Mass Determination of V8 Digest Fragments.

All predicted mass analyses based on primary sequence were done using either General Protein Mass Analysis for Windows (GPMAW), version 4.04, [®]Lighthouse Data, or, Statistical Analysis of Protein Sequences (SAPS)(50), available for internet use at <http://www.isrec.isb-sib.ch/software/SAPS>. Molecular mass determination by SDS-PAGE was extrapolated from pre-stained standards from separate suppliers consisting of either lysozyme, trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, and myosin (H-chain), with respective molecular masses of 18, 28, 39, 60, 84, 120, and 215 kDa, (GIBCOBRL) or, lysozyme, β -lactoglobulin, carbonic anhydrase, ovalbumin, BSA, phosphorylase B, myosin (H-chain) with respective molecular masses of 15, 20, 30, 47, 73, 121, and 216 kDa respectively (Pierce).

Results

This study was designed to isolate and identify proteinase-stable, heme-coordinating regions of human neutrophil flavocytochrome b_{558} . Our approach used limited digestion of partially-purified, detergent-solubilized flavocytochrome b with *Staphylococcal* V8 proteinase. Following digestion, HPLC size exclusion chromatography was used to isolate heme-associated polypeptides. Heme and protein content were simultaneously measured by monitoring the absorbance spectrum from 230 to 600 nm with a UV-visible diode array detector (DAD) connected in series with a

fluorometer ($\lambda_{\text{ex}} = 280\text{nm}$, $\lambda_{\text{em}} = 340\text{nm}$). Figure 2.1 shows a three dimensional chromatogram of partially-purified flavocytochrome *b* (1.6 nmoles heme) during the final HPLC size exclusion purification step prior to digestion with V8 proteinase.

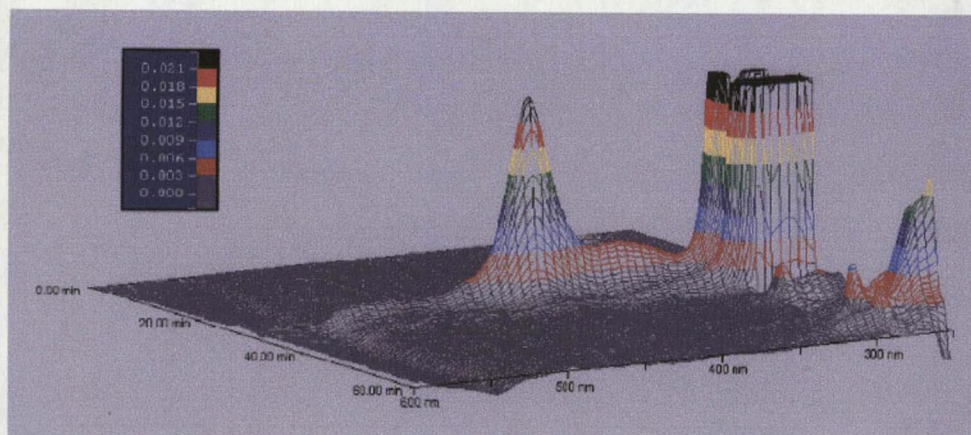


Figure 2.1. HPLC size exclusion purification of detergent solubilized flavocytochrome *b*. A three dimensional UV-Visible diode array spectrophotometer chromatogram of detergent-solubilized, flavocytochrome *b* (1.6 nmoles heme) during the final HPLC size exclusion chromatography step prior to digestion with V8 proteinase, as described under "Experimental Procedures". The axes are: X, elution time (min); Y, absorbance wavelength (nm); and Z, absolute absorbance (AU). Flavocytochrome *b* is the prominent peak eluting at ~ 27 min with an oxidized heme absorbance maximum (λ_{max}) at 414 nm. Coeluting absorbance maxima between 250 and 320 nm derive from protein and Triton X-100. The numbers and their corresponding colors in the upper left of the figure are a scale of absolute absorbances in AU. This chromatogram is typical of

results obtained from at least five separate experiments conducted on different days.

Flavocytochrome *b* is readily identifiable as the prominent 414 nm absorbance peak eluting at 27 minutes, corresponding to an apparent mass of 300-330 kDa relative to soluble size exclusion chromatography protein standards. This apparently anomalous elution mass is independent of the detergent used (Triton X-100, DDM, and OG, with detergent micelle molecular masses of ~ 90, 24, and 18 kDa respectively) for either solubilization or HPLC column buffer (T. Foubert, unpublished observations). Since detergent-solubilized flavocytochrome *b* in similar conditions has been shown to have a 1:1 subunit stoichiometry (21,51,52), the apparent elution mass probably arises from a combination of the asymmetric geometry of the protein (21), and the roughly gram per gram ratio of bound detergent typical of transmembrane proteins (21,53).

Additionally, purification conditions similar to those used in this work have yielded flavocytochrome *b*-Rap1A complexes (36,54). We thus infer that the apparent 300-330 kDa elution mass represents the unit size of monodisperse detergent-bound flavocytochrome *b*, or possibly a flavocytochrome *b*-Rap1A complex. The absorbance peaks between 250 and 320 nm that coeluted with flavocytochrome *b* derive from protein and associated Triton X-100. 400 μ L fractions were collected from the column at 1 minute intervals from consecutive runs, and the peak flavocytochrome *b*-containing fractions that eluted between 25-29 minutes were pooled and digested.

Evolution of Flavocytochrome *b* Heme Absorbance Spectrum During Proteolysis.

Digestion of flavocytochrome *b* for periods up to 4.5 hours produced consolidated, heme-containing peaks during HPLC size exclusion chromatography, while longer digestion periods resulted in a gradual loss of recoverable heme activity. Absorbance measurements taken from three time points during a 4.5 hour digestion of flavocytochrome *b* with V8 proteinase are shown in Figure 2.2, illustrating the evolution of the oxidized flavocytochrome *b* Soret absorbance spectrum.

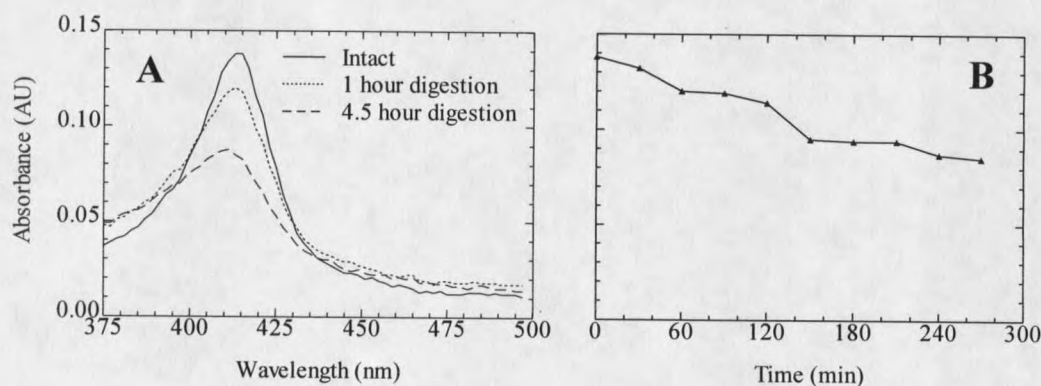


Figure 2.2. Evolution of flavocytochrome *b* heme oxidized Soret absorbance spectrum during proteolysis. Detergent-solubilized, partially-purified flavocytochrome *b* (2.5 nmoles heme) was exposed to V8 proteinase as described under "Experimental Procedures" for a total of 4.5 h. Aliquots were removed from the digestion vessel and their absorbance spectra recorded. A) Oxidized Soret heme absorbance spectra of intact (—), and following 1 (.....) and 4.5 (---) h of digestion. Both intact and 1-h digested flavocytochrome *b* λ_{max} is at 414 nm, and ~ 86% of the heme absorbance is retained after

1 h of digestion. At 4.5 h, 62% of the initial heme absorbance is retained, and $\lambda_{\max}$ is shifted to 408-410 nm. **B)** Triangles represent 414 nm absorbance values measured at 30 min intervals throughout the 4.5 h digestion. These results are typical of values obtained from at least five separate experiments conducted on different days.

Samples were removed at 30 minute intervals from the digestion milieu, placed on wet ice, and supplemented with a proteinase-inhibitor cocktail to inhibit further digestion. The absorbance spectrum of each time point was then recorded, and the absorbance maxima ($\lambda_{\max}$) were plotted over time (Figure 2.2 B). After 60 minutes of digestion, a slight broadening of the Soret peak was observed, while $\lambda_{\max}$ remained at 414 nm with approximately 86% of the starting 414 nm absorbance retained (Figure 2.2 A). By 270 minutes of digestion, the Soret absorbance peak had broadened further, concurrent with a blue shift in $\lambda_{\max}$ from 414 nm to 408-410 nm (Figure 2.2 A), and an overall reduction in the Soret absorbance to 62% of the starting value (Figure 2.2 B).

Isolation of Heme-bearing Proteolytic Fragments of Flavocytochrome *b* After 1 Hour of Digestion.

Samples that were collected from the digestion milieu at thirty minute intervals were again subjected to HPLC size exclusion chromatography to isolate possible heme-associated peptides (Figure 2.3). Prior to digestion, flavocytochrome *b* eluted as a single 414 nm absorbance peak at 27 minutes corresponding to a molecular mass of 329 kDa

relative to soluble globular protein standards (Figure 2.3, Top).

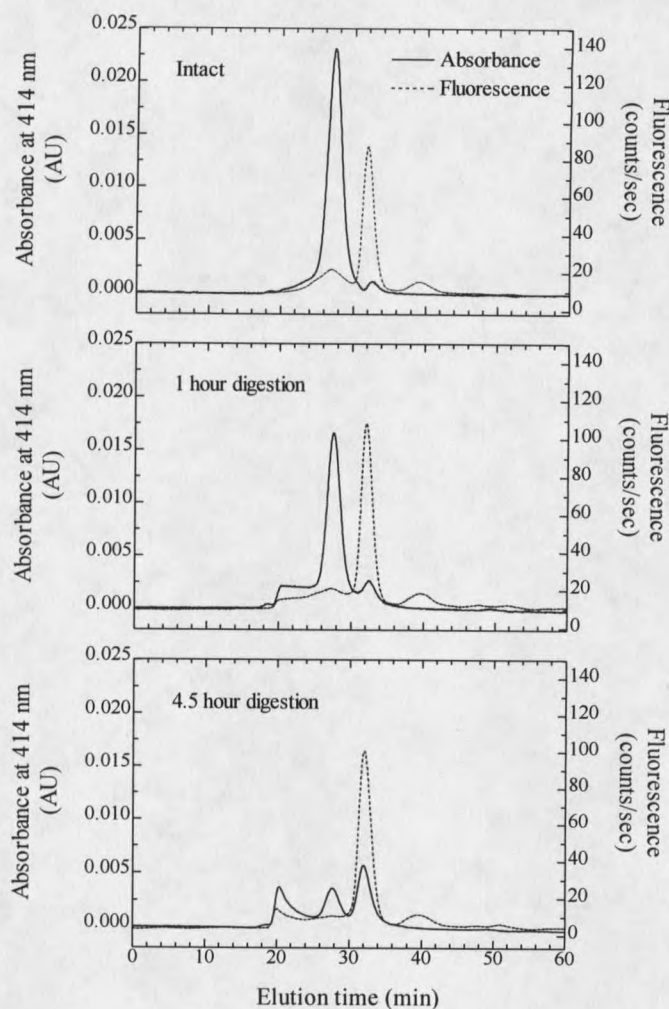


Figure 2.3. HPLC size exclusion chromatograms of flavocytochrome *b* at three time points during 4.5 hour digestion. Detergent solubilized, partially-purified flavocytochrome *b* was digested with V8 proteinase for 4.5 h, aliquots were removed every thirty min and subjected to HPLC size exclusion chromatography as described in “Experimental Procedures”. Shown in each panel are the normalized 414 nm absorbance

(—) and fluorescence (---) ($\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$) chromatograms from samples collected at the indicated time points. **Top.** Intact flavocytochrome *b* elutes as a monodisperse peak centered at 27 min, with λ_{max} at 414 nm, corresponding to a mass of ~ 329 kDa relative to globular size exclusion chromatography standards. The fluorescence peak at eluting at 32 min is due to micellar Triton X-100 from the injection bolus, and corresponds to a mass of ~ 135 kDa. **Middle.** Flavocytochrome *b* at 1 h of digestion. After 1 h of digestion, all of the flavocytochrome *b* heme absorbance is recovered. The integrated 414 nm heme absorbance of the prominent peak is equal to 74% of the corresponding non-digested flavocytochrome *b* peak. The peak elution time is retarded by 0.6 to 0.8 min relative to non-digested flavocytochrome *b*, corresponding to a ~ 30-34 kDa reduction in mass. A small amount of the 414 nm absorbance is redistributed within an aggregate species that eluted between 19-25 min, corresponding to a mass range spanning 1300 to 461 kDa respectively. A smaller heme-associated species began to accumulate in the 32 min elution fraction, coeluting with the micellar Triton X-100. A similar redistribution of the fluorescence intensity was observed up to 30 min of elution, and a 20% increase was observed at 32 min. **Bottom.** Flavocytochrome *b* at 4.5 h of digestion. The 414 nm heme absorbance is distributed between three peaks with an overall loss of 37% relative to non-digested flavocytochrome *b*. The first peak eluting at ~ 20.5 min corresponds to an aggregated species with a mass of ~ 1000 kDa, the second peak at 27 min is the remaining partially-proteolysed 1 h digest fragment, and the third peak at 32 min corresponds to a mass of ~ 135 kDa. The fluorescence intensity profile again paralleled the redistribution of the 414 nm absorbance during the first 30 min of the

elution profile, while the 32 min peak remained constant. The results shown here are typical of at least three separate experiments.

The DAD spectrum (Figure 2.1) revealed that the small absorbance peak centered at 32 minutes elution time was not from heme, but was contributed by the large absorbance shoulder of micellar Triton X-100 from the injection bolus. Consistent with buffer blanks, the high level of fluorescence associated with this elution time was also contributed primarily by micellar Triton X-100. Silver-stained SDS-PAGE gels of this fraction showed only negligible levels of protein (not shown). The small, broad fluorescence peak centered at 39-40 minutes is a contaminant of unknown origin that was also present in buffer blanks.

Digestions for 1 hour consistently produced a prominent heme-associated protein fragment that eluted as a single peak (Figure 2.3, Middle) with a λ_{max} at 414 nm. Integration of the 414 nm absorbance profiles of the intact and 1-hour digested flavocytochrome *b* indicated a 95% recovery of the total heme absorbance after 1 hour of digestion. Comparison of the integrated 414 nm absorbance values of the principal 25-30 minute elution peaks for the intact and 1-hour digested fractions indicated that 74% of the heme absorbance was retained by the digest fragment. The elution time of the peak fraction was retarded ~ 0.6-0.8 minutes relative to nondigested controls, corresponding to a reduced mass of roughly 30-34 kDa from the initial 329 kDa mass. Additionally, the 414 nm absorbance profile began to transform during the first hour of digestion. An aggregated species was observed at an elution time of 19-25 minutes, corresponding to

mass range spanning 1300 kDa to 461 kDa respectively. An increase in the 414 nm absorbance was also observed at an elution time of 32 minutes, suggesting an accumulation of heme or heme-bearing protein fragments that either coeluted with or were partitioned into Triton X-100 micelles. The DAD absorbance spectra of both the aggregated and the smaller species, however, revealed a λ_{max} at 408-410 nm (not shown) suggesting an altered heme environment. The fluorescence intensity profile during the first hour of digestion also exhibited a redistribution trend similar to the absorbance. The fluorescence associated with the prominent absorbance peak eluting at 25-30 min decreased slightly in intensity, concurrent with a slight increase in the aggregate fractions (elution time = 19-25 min), and a 20% increase in the fraction eluting at 32 minutes.

Isolation of Heme-bearing Proteolytic Fragments of Flavocytochrome *b* After 4.5 Hours of Digestion.

Continued digestion beyond 1 hour resulted in a gradual division of the 414 nm absorbance profile into a trimodal distribution that reached a maximum accumulation at 4.5 hours (Figure 2.3, Bottom). Integration of the 414 nm absorbance profiles from this time point indicated that approximately 63% of the total initial heme absorbance was recovered. The first peak to elute at 20.5 minutes corresponds to an aggregated species with a molecular mass of ~ 1000 kDa, and the second peak, centered at 27-28 minutes elution time is the partially-proteolysed intermediate fragment, prominent after 1 hour of digestion (see above). The third peak, coeluting with micellar Triton X-100 at 32

minutes, corresponds to a molecular mass of ~ 140 kDa. The DAD absorbance spectrum of the 32 minute elution peak showed a large shoulder from the Triton X-100, similar to that observed after 1 hour of digestion, but also revealed a distinct λ_{max} at 408-410 nm indicating the presence of heme in these fractions (not shown). The fluorescence associated with the 25-30 minute elution fractions was also reduced, and a continued redistribution to the aggregated species eluting between 20-25 minutes was observed. The fluorescence intensity associated with the 32 minute elution peak remained constant.

Heme Content of HPLC Fractions.

Throughout the 4.5 hour digestion period, the majority of the heme absorbance appeared confined to the prominent peaks that eluted prior to 35 minutes. The heme distribution throughout the elution profile was confirmed using a modified 3,3',5,5'-Tetramethylbenzidine (TMBZ) assay to allow direct heme quantitation of each HPLC fraction (Figure 2.4). The assay also provided a 100-fold increase in heme-detection sensitivity which allowed heme quantitation of HPLC fractions that were otherwise hindered by low absorbance levels, changes in the heme absorbance spectrum, or absorbance interference contributed by Triton X-100. The TMBZ assay profiles (Figure 2.4) indicated that 84% of the heme from intact flavocytochrome *b* (Figure 2.4, Top) eluted prior to 35 minutes, with 71% contained within the prominent peak fractions that eluted between 24 to 30 minutes. Following 1 hour of digestion and HPLC size exclusion chromatography (Figure 2.4, Middle), the heme distribution remained

relatively constant with 85% contained within the fractions that eluted prior to 35 minutes, and 70% contained within the prominent absorbance peak fractions that eluted between 24 and 30 minutes. By 4.5 hours of digestion (Figure 2.4, Bottom), the heme distribution evolved to a primarily trimodal distribution with 73% eluting prior to 35 minutes. Minor heme-containing fractions were also evident, eluting at 37 and 47 minutes.

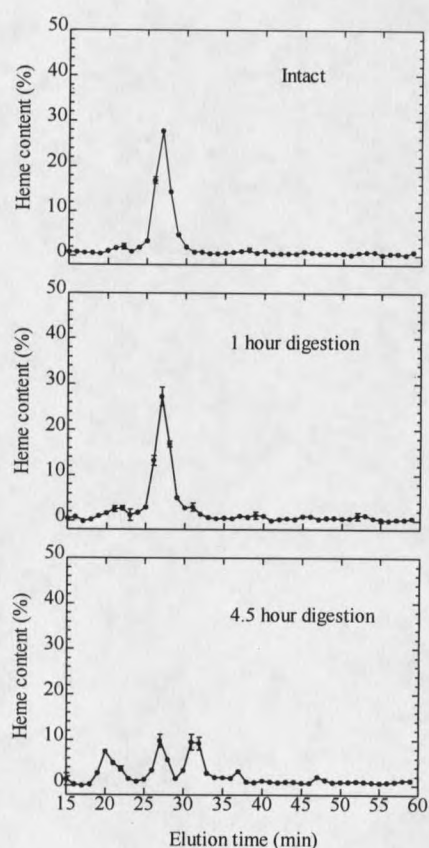


Figure 2.4. Heme distribution of intact and partially proteolysed flavocytochrome *b* after separation by HPLC size exclusion chromatography. 400 μ L fractions were

collected every min throughout the HPLC size exclusion runs shown in Figure 2.3 and analyzed for heme content using a modified TMBZ assay as described under "Experimental Procedures". The heme content of each HPLC size exclusion fraction is shown as a percent of the total heme of the HPLC runs of intact (Top), and following 1 (Middle), and 4.5 (Bottom) h of digestion. Symbols ($\circ$) denote average $\pm$ SD values obtained from two separate TMBZ assays corresponding to the indicated HPLC elution times. **Top.** Intact flavocytochrome *b*. 84% of the total heme is contained within the fractions that eluted prior to 35 min, and 72% of the total heme is contained by the fractions that elute between 24-30 min. **Middle.** Flavocytochrome *b* after 1 h of digestion. 85% of the of the total heme eluted before 35 min, and 70% of the total heme is contained within the 24-30 min elution fractions. **Bottom.** Flavocytochrome *b* after 4.5 h of digestion. 73% of the remaining heme elutes before 35 min, mainly distributed between the three peaks eluting between 18-24, 24-29, and 29-34 min, representing 22, 23, and 28%, respectively.

Identification of Heme-bearing Proteolytic Species by SDS-PAGE and Immunoblotting.

We next identified the polypeptide fragments of flavocytochrome *b* that coeluted with heme spectral activity by separation of the individual HPLC elution fractions on SDS-PAGE, followed by silver staining or immunoblotting with previously characterized (see Methods) flavocytochrome *b*-specific antibodies (Figure 2.5 and Table 2.1). The silver stained gel (Figure 2.5 A, lane 2) and the immunoblot (Figure 2.5 B, lane

1) both show non-digested gp91^{phox} as a diffuse band, centered at ~ 90 kDa molecular mass. Silver staining and immunoblotting of non-digested flavocytochrome *b* with α -p22^{phox} antibodies both show a consolidated band at 22 kDa molecular mass (Figure 2.5 A lane 2, Figure 2.5 B, lane 3 respectively), although the silver stain is less well represented due to its atypical staining characteristics (9). Following 1 hour of digestion, the prominent feature on the silver stained gel was a diffuse, asymmetrically-stained band with a centroid mass between 60 and 66 kDa (Figure 2.5 A, lane 3), and immunoblots of the same fraction exhibited a similar, but more homogeneous staining pattern for gp91^{phox} (Figure 2.5 B, lane 2). The silver stained gel also shows an accumulation of a 17 kDa band (Figure 2.5 A, lane 3) concurrent with an identical shift in the molecular mass of p22^{phox} as indicated by immunoblotting with α -p22^{phox} antibodies (Figure 2.5 B, lane 4). Thus, during the first hour of proteolysis, the average molecular mass of gp91^{phox} was reduced from 90 kDa to 60-66 kDa, and the molecular mass of p22^{phox} was reduced from 22 kDa to ~ 17 kDa. The immunoblots shown in Figure 2.5 B are representative of results obtained from multiple analyses that are summarized in Table 2.1. The information derived from the immunoblot analyses indicated that the epitope regions ³⁸³PKIAVDGP³⁹⁰, ⁴⁹⁸EKDVITGRKQ⁵⁰⁷, and ⁵⁴⁸KQSISNSES GP⁵⁵⁸ of gp91^{phox} and ¹⁸³PQVNPI¹⁸⁸ of p22^{phox} were lost within the first hour of digestion (Table 2.1). The apparent mass reduction of gp91^{phox} during the first hour of digestion did not appear to be due to loss of carbohydrate as indicated by the consonant heterogeneity between the digested and non-digested samples (Figure 2.5 A, compare lanes 2 and 3, Figure 2.5 B, compare lanes 1 and 2).

This observation, in combination with the results of the immunoblotting, suggests that the 60-66 kDa molecular mass proteolytic fragment possesses all of the gp91^{phox} glycosylation sites and provides additional evidence supporting a previous report that proposed ¹³¹N, ¹⁴⁸N, and ²³⁹N as the sites of gp91^{phox} glycosylation (55).

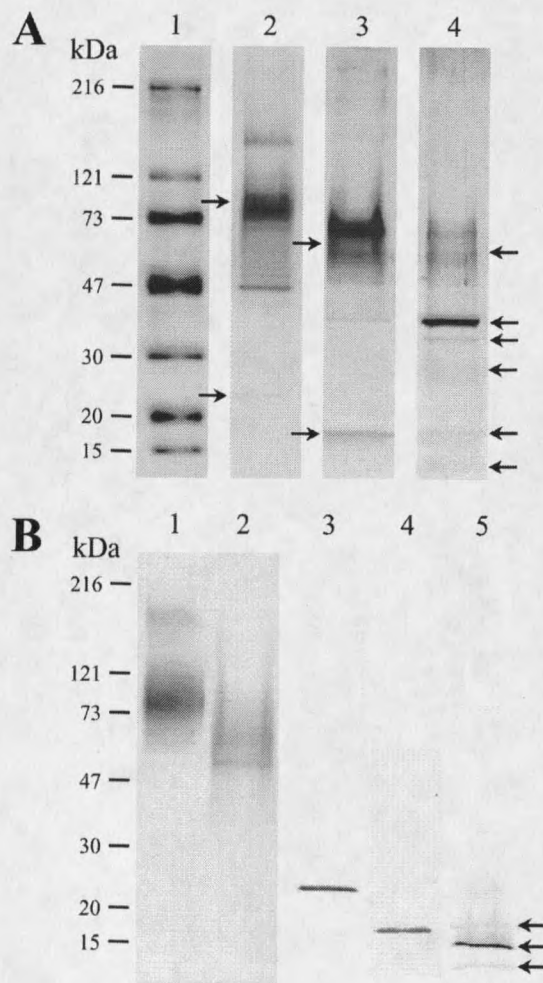


Figure 2.5. Heme-associated proteolytic fragments of flavocytochrome *b* following HPLC size exclusion chromatography. 400 μ L fractions were collected every

min throughout the HPLC runs shown in Figure 2.3. Fractions were separated by SDS-PAGE and either silver stained or transferred to nitrocellulose and immunoblotted with either α -gp91^{phox} (CL5), α -p22^{phox} (NS5 or 44.1) mAbs as described in "Experimental Procedures". **A)** A composite from silver stained SDS-PAGE gels consisting of molecular weight markers (lane 1), intact (lane 2), 1-h digested, 27-28 min elution fraction (lane 3), or 4.5-h digested, 32-33 min elution fraction (lane 4). Arrows indicate intact (lane 2) or prominent digest fragments (lanes 3, 4) of gp91^{phox} and p22^{phox}. **B)** An immunoblot composite of samples corresponding to those shown in Panel A. Intact gp91^{phox} is recognized by mAb CL5 as a diffuse band centered at ~ 90 kDa molecular mass (lane 1). At 1 h of digestion, the large subunit mass is reduced to a ~ 60 kDa diffuse band (lane 2). Intact p22^{phox} is recognized by mAbs 44.1 (not shown), and NS5 (lane 3) as a consolidated band at 22 kDa molecular mass. After 1 h of digestion, p22^{phox} is recognized by mAb NS5 as a band at 17 kDa molecular mass (lane 4). At 4.5 h of digestion, none of the α -gp91^{phox} immunoblots were positive for the 32-33 min elution fraction, but α -p22^{phox} immunoblots were positive for bands at 17, 15, and < 15 kDa molecular mass (lane 5). The immunoblots shown are representative of multiple analyses that are summarized in Table 2.1.

By 4.5 hours of digestion, immunoblots of the 32 minute elution peak fraction (Figure 2.3, lower) using our panel of α -gp91^{phox} antibodies were negative (Table 2.1) even though multiple bands with molecular masses ranging from <15 kDa to ~ 32 kDa were evident by silver staining (Figure 2.5 A, lane 4, arrows). However, immunoblots of

the same elution fraction with α -p22^{phox} antibodies were positive for multiple fragments ranging from 17 to <15 kDa molecular mass (Figure 2.5 B, lane 5).

α -gp91 ^{phox} Antibody	Epitope	1 h digestion molecular mass (kDa)
NL10 (M)	¹³⁵ DPYSVAL ¹⁴¹	60, 17
CL5 (M)	¹³⁵ DPYSVALSELGDR ¹⁴⁷	60
KIS (P)	²⁴⁷ KISEWGKIKE ²⁵⁶	60
54.1 (M)	³⁸³ PKIAVDGP ³⁹⁰	(-)
NL7 (M)	⁴⁹⁸ EKDVITGRKQ ⁵⁰⁷	(-)
KQS (P)	⁵⁴⁸ KQSISNSES GP ⁵⁵⁸	(-)

α -p22 ^{phox} Antibody	Epitope	1 h digestion molecular mass (kDa)
NS5 (M)	⁵⁸ KRKKGSTME ⁶⁶ - ⁷⁸ KLFGPF ⁸³	17, 15
NS2 (M)	¹³¹ WTPIEPKPR ¹³⁹	17
EAR (P)	¹⁶² EARKKPSEEEAAA ¹⁷⁴	17, 16
CS9 (M)	¹⁶⁵ KKPSE ¹⁶⁹	17
44.1 (M)	¹⁸³ PQVNPI ¹⁸⁸	(-)
NS1 (M)	**GIRSXDDSG	(-)
R3179 (P)	Unknown	17

** Conformational epitope
 (-) Epitope not detected by immunoblot
 (M),(P) Monoclonal, Polyclonal antibody

Table 2.1. Immunoblot analyses of partially proteolysed flavocytochrome *b*.

Detergent solubilized, partially-purified flavocytochrome *b* was digested with V8 proteinase for 1 h and subjected to HPLC size exclusion chromatography. The heme-

bearing core polypeptide from the 27-28 min elution fraction shown in Figure 2.5 was analyzed by immunoblot using either α -gp91^{phox} or α -p22^{phox} antibodies as described in "Experimental Procedures". Abbreviations: (M), monoclonal antibody; (P), polyclonal antibody. Molecular masses shown in bold were the predominantly staining species within the particular fraction. The indicated time point samplings are analogous to lanes 2, and 3 of Figure 2.5 A. Immunoblots are from three separate digestion experiments from different days, with each antibody tested twice against positive control lanes (not shown) consisting of aliquots of intact flavocytochrome *b* removed from the final size exclusion purification step prior to addition of V8 proteinase. Superscript numbers denote primary sequence designation with the NH₂-terminal initiation methionine as number 1 of each respective subunit.

The transition from the 1 hour digest heme distribution to the trimodal distribution seen at 4.5 hours of digestion (Figure 2.3) suggested the possibility of a lower molecular mass component that would provide more precise information regarding the heme-ligating regions of flavocytochrome *b*. However, in repeated experiments, the peptide fragments associated with the 32 minute elution peak varied in both composition and quantity. Additionally, the numerous peptides (Figure 2.5 A, lane 4) that coeluted with micellar Triton X-100 at 32 minutes (Figure 2.3) introduced ambiguities that prohibited assignment of heme ligation to specific peptide components.

Identification of Heme-bearing Fragments After 1 Hour of Digestion.

We thus chose to focus our efforts on identification of the polypeptide components present in the 27-28 minute fraction from the 1 hour digestion using a combination of NH₂-terminal sequence analysis, SDS-PAGE, and immunoblotting. Table 2.2 shows the results of the NH₂-terminal sequence analyses, identifying the 1 hour digest 60-66 kDa and 17 kDa molecular mass proteolytic fragments (Figure 2.5 A, lane 3 and Figure 2.5 B, lanes 2 and 4) as the intact NH₂-termini of gp91^{phox} and p22^{phox} respectively. Interestingly, the absence of the initiating methionine on both peptide sequences suggests that they are removed during the maturation of the protein. This result confirms our previous observations (9), but contrasts two previous reports of modification of the NH₂-terminus of the small subunit (56,57).

It is interesting that the contaminating proteins present in the partially-purified flavocytochrome *b* (Figure 2.5 A, lane 2) that was used for the subsequent digestion steps did not interfere with our ability to obtain NH₂-terminal sequence data following proteolysis. After the first hour of digestion, silverstained SDS-PAGE gels indicate that the majority of the signal is consolidated to only two bands (Figure 2.5 A, lane 3, arrows). The NH₂-terminal sequence data (Table 2.2) was obtained by excising the entire diffuse band from ~ 50 to 70 kDa, and the consolidated band at 17 kDa for analysis. Since the NH₂-terminal sequencing process is exquisitely sensitive to background signal from contaminating protein, the ability to obtain unambiguous sequencing data suggests that the additional proteins present during the initial purification step were more

susceptible to proteolysis than flavocytochrome *b*.

Protein Match	Amino Acid Sequence
gp91 ^{phox}	² GNWAVNEGLS ¹¹
p22 ^{phox}	² GQIE-AM-AN ¹¹

Table 2.2. NH₂-terminal sequences of V8 proteolysed flavocytochrome *b* digested for 1 hour with V8 proteinase. Detergent solubilized, partially-purified flavocytochrome *b* was digested with V8 proteinase and subjected to HPLC size exclusion chromatography. Elution fractions that retained heme absorbance were pooled and prepared for NH₂-terminal sequencing as described under "Experimental Procedures". The polypeptides transferred to PVDF membrane for sequencing were analogous to those shown in Figure 2.5 A, lane 3. The discrete band at 17 kDa molecular mass and the entire broad band from ~ 50-70 kDa molecular mass were excised separately and analyzed by NH₂-terminal sequence analysis. Superscript numbers denote primary sequence designation with the NH₂-terminal initiation methionine as number 1 of each respective subunit, and dashes indicate non-matching residues. Of the ten cycles requested for each analysis, 10 of 10 residues from the 60-66 kDa molecular mass fragment matched the NH₂-terminal end of gp91^{phox}, and 8 of 10 residues from the 17 kDa molecular mass fragment matched the NH₂-terminal end of p22^{phox}.

Mass Determination of gp91^{phox} and p22^{phox} Digestion Fragments.

Identification of the COOH-terminal proteinase cut sites of the 1-hour digested, 17 kDa and 60-66 kDa molecular mass fragments was first investigated using SDS-PAGE analysis. To circumvent the anomalous migration behavior of glycosylated, integral-membrane proteins on SDS-PAGE, accurate mass determination of the peptide components from the 27-28 minute elution peak from the 1 hour digestion (Figure 2.5 A, lane 3) required that we first deglycosylate, then reduce and alkylate the proteolytic fragments prior to SDS-PAGE analysis. Enzymatic deglycosylation of intact gp91^{phox} with PNGase F, followed by reduction and alkylation prior to separation by SDS-PAGE, resulted in the appearance of multiple discrete bands by immunoblot (Figure 2.6 A, lane 4). The smallest species (Figure 2.6 A, lane 1, arrow) corresponded to an apparent mass of 60-66 kDa, thus consistent with the predicted mass of the polypeptide core of gp91^{phox} based on primary sequence (33,35). Similarly, immunoblots of nondigested gp91^{phox} that were deglycosylated using chemical methods (see Methods), followed by reduction and alkylation, showed only a single consolidated band of the same mass (not shown). Although SDS-PAGE migration of the deglycosylated core polypeptide of gp91^{phox} has been reported previously with apparent molecular masses ranging from 49 to 58 kDa (9,58),(59) these reports did not include reduction and alkylation following deglycosylation, suggesting that inclusion of these steps promotes more complete unfolding of the protein during SDS-PAGE separation. Reduction and alkylation of non-digested flavocytochrome *b* resulted in a slight increase in the apparent molecular mass

of gp91^{phox} (Figure 2.6 A, compare lanes 2 and 3), but had no effect on the electrophoretic migration of p22^{phox} (Figure 2.6 B, compare lanes 2 and 3). However, simultaneous exposure of p22^{phox} to the conditions used for enzymatic deglycosylation of gp91^{phox}, followed by reduction and alkylation, resulted in a slight increase in apparent molecular mass (Figure 2.6 B, lane 4).

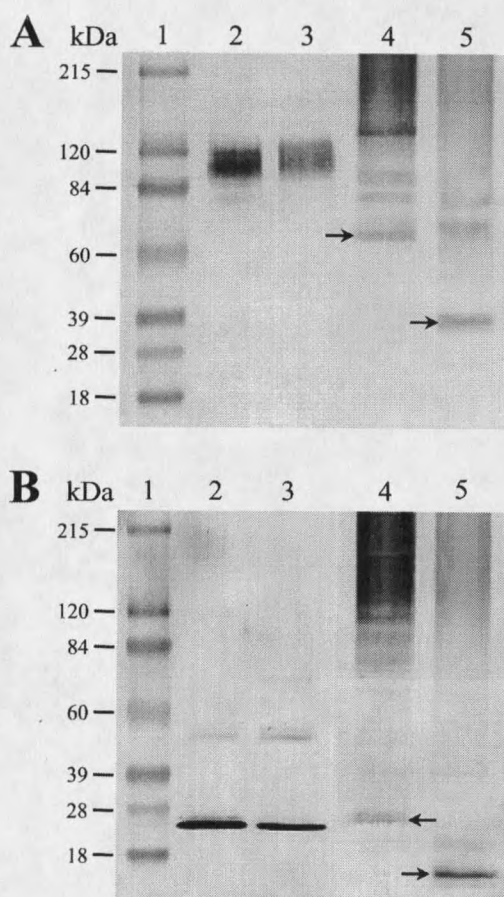


Figure 2.6. Mass determination of deglycosylated fragments of flavocytochrome *b*. Intact flavocytochrome *b*, and polypeptides isolated from the 1 h digest, 27-28 min HPLC elution fraction, were deglycosylated, reduced and alkylated, separated by SDS-

PAGE, and immunoblotted using either α -p22^{phox} or α -gp91^{phox} primary antibodies as described in "Experimental Procedures". **A)** α -gp91^{phox} immunoblots (mAb CL5) of flavocytochrome *b* samples showing, prestained MW markers (lane 1), intact, non-reduced and alkylated (lane 2), intact, reduced and alkylated (lane 3), intact, deglycosylated, reduced and alkylated (lane 4), and 1-h digested, deglycosylated, reduced and alkylated (lane 5) flavocytochrome *b*. **B)** α -p22^{phox} immunoblots of samples identical to Panel A with primary mAb NS1, lanes 2 and 3, or mAb NS5, lanes 4, and 5. Arrows in both panels indicate lowest apparent molecular mass species following deglycosylation, reduction, and alkylation.

Identical treatment of the 1-hour digested polypeptide core had no effect on the electrophoretic migration of the 17 kDa proteolytic fragment of p22^{phox} (Figure 2.6 B, lane 5). Thus, after 1 hour of digestion, the remaining heme-bearing core polypeptide component of flavocytochrome *b* is comprised of fragments of both gp91^{phox} and p22^{phox}, with respective molecular masses of 39 and 17 kDa. Mass predictions from the primary sequence of gp91^{phox}, less the start methionine, suggest that the most likely C-terminal proteinase target sites that correspond to the apparent 39 kDa molecular mass are E³²⁰, E³³⁶, E³⁴⁸, and E³⁶³, yielding masses of 36.7, 38.5, 39.9, and 41.7 kDa respectively.

Similarly, beginning at the NH₂-terminus for p22^{phox} less the start methionine, the most likely cut site for the proteinase corresponding to the 17 kDa band would be at E¹⁶², yielding a predicted mass of 17.7 kDa. However, the positive immunoblot signal from both the polyclonal anti-peptide EAR, ¹⁶²EARKKPSEEEAAA¹⁷⁴, and the mAb CS9,

$^{165}\text{KKPSE}^{169}$, antibody (Table 2.1) indicate that the 17 kDa molecular mass fragment retains at least part of these epitopes. The next most likely proteolytic cleavage site lies within the glutamate repeats E^{169} to E^{171} , yet cleavage within this site would yield predicted masses of 18.5 to 18.8 kDa, not the 17 kDa mass observed by SDS-PAGE. Since the mAb 44.1 epitope region, $^{183}\text{PQVNPI}^{188}$, (Table 2.1) is removed during the first hour of digestion, and there are no other Glu residues C-terminal to E^{171} , we conclude that the V8 cleavage site resides within the glutamate repeats E^{169} to E^{171} and are uncertain as to the anomalous migration behavior of the p22^{phox} fragment on SDS-PAGE. In conclusion, the combined results of the antibody, NH_2 -terminal sequence, and SDS-page analyses, identify the heme-bearing proteolytic polypeptide core as the NH_2 -terminal 320 to 363 amino acids of $\text{gp91}^{\text{phox}}$ and the NH_2 -terminal 169 to 171 amino acids of p22^{phox} .

Discussion

The principal conclusion of this study is that a flavocytochrome *b* heme-binding domain resides within a polypeptide core region consisting of the NH_2 -terminal 320 to 363 amino acids of $\text{gp91}^{\text{phox}}$ and the NH_2 -terminal 169 to 171 amino acids of p22^{phox} . The fragment, isolated by exposure of partially purified flavocytochrome *b* to *Staphylococcal* V8 proteinase and size exclusion chromatography, was shown to retain a native heme absorbance spectrum.

Our initial digestion experiments were conducted for periods of up to 18 hours to establish conditions rendering the smallest proteolytic fragments of flavocytochrome *b* that still retained native spectral activity and were separable by size exclusion chromatography. Digestion for 1 hour reduced the mass of the flavocytochrome *b* by roughly 35% but produced a fragment that was spectrally stable for days at 4° C in detergent-containing buffer. Longer digestion periods destabilized the heme environment as evidenced by blue-shifted Soret maxima and an inability to reproducibly recover smaller heme-associated fragments. Interestingly, free hemin introduced to identical HPLC size exclusion chromatography conditions, with either OG or Triton X-100-containing column buffers, coeluted with the detergent micelles (not shown), and displayed a λ_{max} of 396 nm, not the observed 408-410 nm. These observations suggest that the non-native heme absorbance spectrum associated with the more digested fractions may derive from heme that remains ligated to a peptide component, but with a modified ligand environment. Although the intrinsic hydrophobic nature of the heme molecule could promote random interactions with the hydrophobic peptide regions of the digested flavocytochrome *b*, such an association would not serve to explain the λ_{max} at 408-410. The possibility of a spurious histidine-heme ligation is also unlikely given the relatively low association constants ($5 \times 10^3 \text{ M}^{-1}$) observed for reconstituted polypeptide maquettes (60).

The data presented herein are consistent with previous findings implicating specific regions within the NH₂-terminal half of flavocytochrome *b* with heme ligation. Examination of human mutations responsible for CGD revealed that the largest number

of lesions occurred within the NH₂-terminal half of gp91^{phox}, including substitutions at His¹⁰¹ and His²⁰⁹ (24). Fujii *et al.* (25,26) noticed a pyridine-induced shift in the ESR spectrum of flavocytochrome *b* towards that of cytochrome P450 of *Pseudomonas putida*. X-ray crystallographic data from cytochrome P450 from *P. putida* (31) showed the hemes coordinated by a species-conserved motif, FXXGXXXCLG, a sequence similar to residues F⁷⁸XXGXXXC⁸⁵ of gp91^{phox}. The proximity of this heme-coordinating motif to His¹⁰¹ and His²⁰⁹ suggested that they were the most likely candidates for the heme ligands of the flavocytochrome *b*. Another His¹⁰¹ → Tyr mutation in gp91^{phox} (23) resulted in a complete loss of the heme absorbance spectrum without affecting flavocytochrome *b* expression. In a separate CGD case, stemming from an Arg⁵⁴ → Ser mutation in gp91^{phox}, flavocytochrome *b* expression levels were unaffected but changes in the dithionite-reduced Soret absorbance spectrum were observed (61), suggesting that the mutation had a direct effect on the heme environment. Interestingly, a heme-coordinating motif, T¹⁵XXLAVHXXXV²⁵, originally found in the β-subunit of cytochrome *b*₅₅₉ of *Synechocystis* 6803 photosystem II (32), is similar to the T²³²XXXLAVH²³⁹XXXV²⁴³ region of human neutrophil gp91^{phox}. The stretch encompassing this region is predicted by hydropathy analysis to be relatively hydrophilic. This prediction is supported by the presence of the purported glycosylation site (55) immediately adjacent to His²³⁹ at N²⁴⁰. Also, the region is flanked by two previously characterized antibody epitope regions, ²²⁶RIVRG²³⁰ of mAb 7D5 (62), and ²⁴⁷KISEWGKIKE²⁵⁶ of polyclonal antibody KIS (Table 2.1), both of which were shown to be accessible on the extracellular aspect of intact human neutrophils. Thus, if His²³⁹ is solvent accessible, it would probably not be

suitable for heme ligation based on thermodynamic considerations (18). The possibility that $p22^{phox}$ alone coordinates a heme is unlikely due to spectroscopic data that implies a bis-histidyl ligation scheme (25,26,63,64) and the presence of only a single invariant histidine, His⁹⁴ (65-67). Since the $gp91^{phox}$, $p22^{phox}$ subunit stoichiometry has been determined to be 1:1 (21,51,52), $p22^{phox}$ would be able to provide only a single histidine for heme ligation. Therefore, heme coordination by His⁹⁴ of $p22^{phox}$ would necessitate that the second histidine ligand be provided by $gp91^{phox}$ (51,68), thereby ligating the large and small subunits. The possibility that $p22^{phox}$ is involved with heme ligation is supported by our previous observations showing that heme remains associated with both $p22^{phox}$ and $gp91^{phox}$ following separation on LDS-PAGE at 4° C (69). We have also shown previously that the subunits of flavocytochrome *b* are separable only under denaturing conditions that result in loss of the heme absorbance spectrum (21), and most recently in collaborations with other colleagues, that the processing and maturation of flavocytochrome *b* expressed in X-CGD promyelocytic leukemia cells requires heme incorporation as a prerequisite for heterodimer assembly. In the present work, the 1 hour digest spectrally stable core polypeptide was heterodimeric. Additionally, the chromatographic distribution of a non-native heme absorbance spectrum correlated well with the presence of His⁹⁴-containing fragments of $p22^{phox}$. Thus, the correlation between the presence of heme and a heterodimeric structure suggests that both subunits contribute to the stability of the heme environment. The spectral stability of the 1 hour digest fragment further suggests that the primary interfacial contact regions between $gp91^{phox}$ and $p22^{phox}$ remain intact within the polypeptide core.

Although transgenic expression of fully processed, spectrally similar gp91^{phox} was demonstrated in the absence of p22^{phox} in two non-promyelocytic cell lines, monkey kidney COS-7 cells and murine 3T3 fibroblasts (70) (personal communication, M. C. Dinauer), the reduced Soret band absorbance spectrum from COS-7 gp91^{phox} alone was similar, but not identical to either cells expressing both subunits, or native human neutrophil flavocytochrome *b*. Moreover, in the absence of co-expressed COS-7 p22^{phox}, COS-7 gp91^{phox} was unable to produce superoxide (71). These observations, combined with our data, thus suggest that the presence of p22^{phox} is probably necessary for stabilization of the native heme environment of flavocytochrome *b*.

Native flavocytochrome *b* contains 19 histidines, 6 to 8 of which are removed during the first hour of digestion depending on the precise C-terminal cleavage site of gp91^{phox}. Since the 1-hour digested polypeptide core retains 74% of the heme absorbance of native flavocytochrome *b*, the C-terminal domains of the protein are probably not involved with heme ligation. Of the 11 to 13 histidines that remain within the 1-hour digested core polypeptide, the polymorphic His⁷² of p22^{phox} (65)-(66,67) is ruled out and the extracellular His²³⁹ of gp91^{phox} is probably an unlikely candidate for heme ligation. Therefore, the remaining 9 to 11 histidines, including His⁹⁴ of p22^{phox}, are the most likely candidates for this function. This number prohibits assignment of specific histidines for the ligation, however, their close proximity to the predicted transmembrane spanning regions of flavocytochrome *b* (33-35) suggests a transmembrane or juxtamembrane heme placement.

In conclusion, this study provides direct evidence that the regions of flavocytochrome *b* composed of the NH₂-terminal 320 to 363 amino acids of gp91^{phox}, and the NH₂-terminal 169 to 171 amino acids of p22^{phox} are responsible for heme coordination. Both regions encompass the predicted transmembrane spanning domains of each subunit, and appear to retain the glycosylation sites for gp91^{phox} as well as the sites of interaction between p22^{phox} and gp91^{phox}.

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CHAPTER 3

STRUCTURAL CHANGES ARE INDUCED IN HUMAN NEUTROPHIL CYTOCHROME *b* by NADPH OXIDASE ACTIVATORS LDS, SDS, and ARACHIDONATE: *REAL TIME INTERMOLECULAR RESONANCE ENERGY TRANSFER BETWEEN TRISULFOPYRENYL-WHEAT GERM AGGLUTININ AND CYTOCHROME b_{558}* .

Introduction

Neutrophils and other phagocytes of the immune system participate in protection of the host by engulfing and destroying pathogens (1-4). Central to this process is the assembly of the NADPH oxidase complex at the neutrophil plasma membrane. Currently, the oxidase is known to consist of the cytosolic proteins, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, the membrane associated Rac1/2, Rap1A, and a transmembrane component, flavocytochrome *b* (5-10). Flavocytochrome *b* is a multi-heme, heterodimeric protein comprised of an extensively glycosylated 91 kDa large subunit, $gp91^{phox}$, and a 22 kDa non-glycosylated small subunit, $p22^{phox}$ (11). Intracellular binding sites on flavocytochrome *b* exist for both FAD (12-15), and NADPH (16,17). Flavocytochrome *b* serves as a locus for assembly of the cytosolic components at the plasma membrane and also functions as the terminal electron carrier for reduction of extracellular molecular oxygen to the highly reactive superoxide anion (O_2^-) (5,18,19). The intrinsic non-covalently bound heme moieties (20) of flavocytochrome *b* appear to be localized to the NH_2 -terminal transmembrane domain (21-23) and are believed to be essential for transmembrane electron transfer (20,24-27) as well as proton conduction (22).

The importance of a functioning NADPH oxidase in the host immune response is demonstrated by individuals with chronic granulomatous disease (CGD). The phagocytes of these individuals are unable to mount an oxidative burst and therefore do not produce superoxide. In the absence of superoxide, the ability of the phagocytes to effectively kill invading organisms is severely diminished, resulting in recurrent, life-threatening infections. The underlying cause of the disease was determined to be a defective NADPH oxidase resulting from genetic anomalies in any one of the several oxidase subunits (28-33). While this knowledge has led to the identification of the multiple components of the oxidase, their physiological roles in control of oxidase activity *in vivo* remain unclear.

Signal transduction events that precede oxidase activation have been identified through the use of the cell-free oxidase assays and other *in vitro* studies. Structural changes in the cytosolic components imparted by phosphorylation, or from interactions with arachidonate (AA), or sodium dodecyl sulfate (SDS), have been shown to encourage or disrupt protein-protein interactions between the oxidase subunits (34-43). Studies of the electron transfer pathway have provided information suggesting that oxidase control points exist between NADPH and FAD (44-47), between FAD and the heme centers (48-51), and at the level of the hemes (44,46,48,51). A precise description of how the culmination of these signal transduction events induces oxidase activation has not been provided.

In vivo, interactions between the cytosolic components of the oxidase, cofactors, and lipids are less clear but have been postulated to act in concert to alter the spatial

relationships between the cofactors, hemes, and the side chains of vicinal aromatic amino acids of flavocytochrome *b*. The resulting active-state geometry probably enables through-protein electron tunneling (52-56). To date however, the induction of an active state geometry of flavocytochrome *b* has not been demonstrated directly. Indirect support for this posit is provided by atomic force microscopy (AFM) studies (57) where conformational changes in cytochrome *b* were inferred based on changes in liposome size during the AA-mediated transition from the inactive to the active state. Other studies have shown that the accessibility of the flavocytochrome *b* hemes to chemically-reactive compounds such as phenyl arsine oxide (PAO) was influenced by SDS and AA in parallel with a transition in the spin-state of the heme iron (58). These effects were shown to be concurrent with the activation state of the oxidase, both in cell-free systems utilizing membrane-bound flavocytochrome *b*, and intact neutrophils (26,59). Further analysis suggested the presence of an amphiphile binding site on flavocytochrome *b* that, once occupied, simultaneously decreased the affinity of PAO for the hemes of flavocytochrome *b* and increased the affinity of NADPH (60). In a separate kinetic study of oxidase activation by SDS and AA (61), Cross and coworkers proposed that the lag in activity during the transition from what appeared to be an intermediate state of oxidase activation to the fully activated state could be explained by a change in the conformation of cytochrome *b*.

Direct interactions between the oxidase activators and flavocytochrome *b* have been inferred from kinetic studies of cell-free systems. The lag period prior to the onset of oxidase activity was shown to decrease when cytosol and membrane fractions were

preincubated together with anionic activators, but not when incubated separately (61-63). Independently, Doussi re and coworkers observed that preincubation of membrane fractions with AA increased both the rate and amount of superoxide produced, while preincubation with the cytosolic fraction alone actually impeded oxidase activation (60). Lastly, Koshkin and Pick and later Doussi re *et al.* showed that purified, relipidated flavocytochrome *b* in the presence of NADPH and FAD sustained limited activator-mediated superoxide production in the absence of the cytosolic subunits (47,60). Collectively, these observations suggest that the amphiphiles interact directly with flavocytochrome *b* to regulate oxidase activity.

In parallel with oxidase activity, a substantial body of evidence indicates that gp91^{phox} also functions as a proton channel. This function provides a necessary compensatory mechanism for maintenance of transmembrane electroneutrality and intracellular pH during oxidase activity (64-66). Proton channel activity appears to be mediated by AA (67-69) that is liberated from phospholipids by the action of cytosolic phospholipase A₂ (cPLA₂). Most recently, proton channel activation has been proposed to be directly linked to release of heme from one of its putative ligands, His¹¹⁵ of gp91^{phox} (22). Changes in histidine ligation during oxidase activation might therefore provide a mechanism that would couple electron and proton transport activities.

To directly test the hypothesis that the oxidase activators mediate the induction of an active-state geometry of flavocytochrome *b*, it would be desirable to have a real-time probe that would be sensitive to heme orientation and placement within the protein. To this end, we utilized wheat germ agglutinin (WGA), conjugated with the acetyl azide

derivative of the trisodium salt of 1,3,Trisulfo-8-pyrenyloxyacethydrazide, or Cascade Blue[®] acetyl azide (CCB), (70) as an extrinsic fluorescence donor molecule (CCB-WGA). Immobilized WGA is a highly effective affinity matrix for purification of detergent-solubilized, neutrophil cytochrome *b* (11). WGA also binds directly to the glycosylated large subunit of cytochrome *b* when transferred to nitrocellulose following separation by SDS-PAGE (71). This lectin-carbohydrate affinity should promote formation of a complex that would allow the intrinsic cytochrome *b* hemes to be utilized as acceptors for resonance energy transfer (RET) from the CCB-WGA. Changes in spatial relationships between these donor-acceptor pairs could then be monitored by RET spectroscopy.

Our results indicate that the CCB-WGA conjugate is able to form a complex with the gp91^{phox} glycan in solution and partially quench the probe fluorescence. This quenching is relaxed by addition of the activators SDS, LDS, or AA, suggesting that they interact directly with flavocytochrome *b* to impart changes in the molecular geometry of the protein. Moreover, these effects approached saturation at activator concentrations consistent with maximal activity in cell-free oxidase assays, and did not occur when the methyl ester of arachidonate (AA-ME), an oxidase antagonist (58,60,72-74), was substituted for the activators in our RET system. Collectively, these observations suggest that the structural changes induced in cytochrome *b* by the activators are functionally-relevant and may therefore represent a necessary component of oxidase or proton channel activation.

Abbreviations

The abbreviations used are: AA, arachidonic acid, arachidonate; AA-ME, arachidonate methyl ester; CCB, Cascade Blue®; CGD, chronic granulomatous disease; CMC, critical micelle concentration; CNBr, cyanogen bromide; DAG, diacylglycerol; GlcNAc, N-acetylglucosamine; NeuNAc, sialic acid, N-acetyl-neuraminic acid; O₂⁻, superoxide anion; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PAO, phenyl arsine oxide; *phox*, phagocyte oxidase; RET, resonance energy transfer; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin.

Experimental Procedures

Materials.

KCl, NaCl, EDTA, NaN₃, gelatin, [ethylenebis(oxyethylenenitrilo)]Tetra acetic acid (EGTA), syringe filters for filtering buffers for fluorescence work, (Whatman, 25 mm diameter, polyethersulfone membrane, 0.2 µm pore size), and sulfuric acid were purchased from Fisher Scientific (Tustin, CA). N-acetylglucosamine (GlcNAc), N-N'-diacetylglucosamine (chitobiose), Heparin-Sepharose 4B beads, N-formyl-Met-Leu-Phe, dihydrocytochalasin B, Na₂ ATP, chymostatin, wheat germ agglutinin (WGA), diisopropylfluorophosphate (DFP), sodium dithionite, quinine sulfate, arachidonate or the methyl ester suspended in chloroform, bovine serum albumin (BSA), fetuin, ovalbumin

(grade VI), proteinase K, HANKS balanced salts, TRIZMA-HCl (Tris-HCL), 30% hydrogen peroxide, ABTS, lithium dodecyl sulfate (LDS), goat α -mouse IgG conjugated to HRP, and Triton X-100 detergent were from Sigma Chemical Co (St. Louis, MO). [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] (HEPES) and ultrapure SDS were obtained from United States Biochemical (Cleveland, OH). N-octyl- β -D glucopyranoside (OG, octylglucoside), dithiothreitol (DTT, Cleland's reagent), and phenylmethylsulfonyl fluoride (PMSF) were from Calbiochem-Novabiochem Corp (La Jolla, CA). CNBr activated Sepharose 4B beads were purchased from Pharmacia Biotech AB (Uppsala, Sweden). 6 mm outside diameter, cylindrical, UV-transparent microcuvettes were purchased from Sienco, Inc (Färgelanda, Sweden). CCB-WGA conjugate was purchased in lyophilized form from Molecular Probes (Eugene, OR). The BCA protein assay, supplied in kit form, and Reacti-Bind[®] 96-well EIA polystyrene plates were from Pierce, Inc (Iselin, NJ).

Buffers.

Buffer A consisted of 150 mM NaCl, 10 mM Na₂PO₄, 0.72 mM Triton X-100 (0.045%, or 3X CMC), pH 7.4 and 0.02% NaN₃, in deionized water, filtered through a 0.2 μ m pore size membrane filter. Membrane resuspension buffer, MRB(-), was made as per the method of Parkos, *et al.* (11), and consisted of 10 mM NaCl, 100 mM KCl, 10 mM HEPES, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4, in deionized water. MRB(+) was MRB(-) supplemented with 1 mM PMSF and 10 μ g/mL chymostatin immediately

before use. 10 mM phosphate buffered saline (PBS) contained 10 mM NaH_2PO_4 , 150 mM NaCl, 0.02% NaN_3 , pH 7.4, in deionized water. Digest buffer contained 50 mM Tris-HCl, pH 7.4, 10 mM MgCl_2 , 0.02% NaN_3 in deionized water. HANKS balanced salt solution was purchased as a premeasured dry powder and mixed as per the manufacturers instructions. Relax(+) consisted of 10 mM NaCl, 100 mM KCl, 10 mM HEPES, 3.5 mM MgCl_2 , and 1.0 mM ATP, pH 7.4. Buffers used for ELISA experiments were: 100 mM phosphate Coupling Buffer (100 mM NaH_2PO_4 and 150 mM NaCl in distilled water, pH 7.2), Blocking Buffer (100 mM phosphate buffer supplemented with 0.05% Tween 20 and 3% BSA), Wash Buffer (10 mM HEPES, 150 mM NaCl in distilled water, pH 7.4), and ABTS reagent (50 mM sodium citrate in distilled water, pH 4.2 supplemented just before use with ABTS and hydrogen peroxide to final concentrations of 455 μM and 0.1% respectively).

Purification of Cytochrome *b*.

Isolation of human PMNs and purification of cytochrome *b* was carried out as described by the method of Parkos *et al.* (11). Cytochrome *b* heme quantitation prior to heparin purification was determined from the reduced *minus* oxidized spectrum at 558 nm using $\Delta\epsilon_{558} = 29.3 \text{ (mM cm)}^{-1}(75)$, blanked against control buffer, and reduced by addition of sodium dithionite, mixed in deionized water immediately prior to use, to a final concentration of 10 mM. After heparin purification, cytochrome *b* heme quantitation was carried out using $\epsilon_{414} = 130.8 \text{ (mM cm)}^{-1}(75)$ blanked against buffer.

The absorbance was measured on either a Hewlett Packard HP 8452A diode-array spectrophotometer or a Molecular Dynamics Spectra-Max 250, environmentally controlled, micro titer plate spectrophotometer. When necessary, the samples were sonicated using a Fisher 50 probe style Sonic Dismembrator, model XL2005.

CCB-WGA Probe.

The CCB-WGA probe was stored in the dark either in lyophilized form at -20°C with desiccant, or suspended at 1 mM concentration in 10 mM PBS, pH 7.4, aliquoted, stored at -20°C , and thawed immediately prior to use. Quantitation of CCB-WGA was accomplished using $\epsilon_{400} = 31,400 \text{ (cm M)}^{-1}$ (Molecular Probes) for the CCB, and the WGA content back-calculated using the labeling stoichiometry average of 4.6:1, CCB:WGA (information supplied by Molecular Probes). Immediately prior to use, the probe was diluted in Buffer A to a working concentration of 10 or 20 nM, based on WGA content, and filtered through a $0.2 \mu\text{m}$ pore size syringe filter to remove particulate contamination. To prevent moisture condensation on cuvettes, the working solution was brought to room temperature before use.

Arachidonate.

AA and AA-ME stocks in chloroform were stored at -20°C , in a sealed, dark container that had been purged with argon. Working solutions were prepared by

transferring aliquots from the stock to a fresh tube where the chloroform was removed by evaporation in a stream of ultra high purity argon at room temperature. The amphiphiles were resuspended in deionized water by brief probe sonication, purged with argon, and kept on ice prior to use. All working solutions of AA and AA-ME were used the same day as prepared.

Fetuin.

The protein was weighed dry, suspended in buffer A, and the concentration determined using the BCA method as described by Pierce, compared to bovine serum albumin standards. Fetuin was prepared as an affinity ligand for the CCB-WGA probe as described previously (76) except CNBr-activated Sepharose 4B beads were purchased from Pharmacia Biotech AB, and the ligand was coupled at 4 mg/mL as per the manufactures instructions.

ELISA.

Assays were conducted in 96-well maleic anhydride-activated polystyrene Reacti-Bind[®] plates. A 100 μ L volume containing 2-10 μ g of WGA or CCB-WGA dissolved in 100 mM phosphate Coupling Buffer was added to each well and allowed to react overnight at room temperature (The amount of WGA or CCB-WGA that bound to the wells was determined by BCA assay to saturate at ~ 0.5 μ g/well for both ligands). The

wells were emptied and blocked by exposure of each well to 200 μ L of Blocking Buffer for two h at room temperature. The wells were then rinsed 5 times with 250 μ M LDS in Buffer A to displace any weakly-bound ligand, followed by 5 rinses with Buffer A. Partially-purified cytochrome *b*, 0.2-0.5 μ g heme, (above) in 100 μ L of Buffer A was then added to each well and incubated overnight at 4° C. The plate was then rinsed 5 times with Buffer A and the different concentrations of LDS or AA dissolved in Buffer A were added. The plate was allowed to incubate for 20 min at room temp, and then rinsed 5 times with Buffer A. 100 μ L of primary antibody, mAb 54.1 (77) at a concentration of 2 μ g/mL in Blocking Buffer was added to each well. The plate was then incubated at room temperature for 2 h and rinsed 5 times with Wash Buffer. 100 μ L of goat α -mouse horse radish peroxidase conjugated IgG secondary antibody was added per well at a 1:1000 dilution in Blocking Buffer and incubated for 1 hour at room temperature. The plate was then rinsed 5 times with Wash Buffer and developed by adding 100 μ L of ABTS reagent per well. Absorbance values were measured at 405 nm.

Fluorescence Measurements.

All reagents containing CCB-WGA were kept in foil-wrapped containers and solutions containing cytochrome *b* or lipids were kept on ice prior to use. Experiments were conducted at ambient temperature in cylindrical 6 mm outside diameter 500 μ L continuously-stirred UV transparent microcuvettes. Fluorescence measurements were

recorded using a Photon Technologies Inc. Quanta-Master QM-1 steady state fluorometer with excitation monochromators set at 376 nm and the emission monochromator set at 418 nm for kinetic measurements. The slit width for both monochromators were set at 2 mm, corresponding to a 4 nm and 8 nm bandpass for the excitation and emission monochromators respectively. All fluorescence spectra were corrected for the wavelength-dependent instrument response using the software functions provided by the manufacturer (Felix software ver 1.1, 1996 PTI, Inc). For all experiments the CCB-WGA working solution (10 or 20 nM) was introduced to a continuously-stirred cuvette, and the fluorescence monitored for a short period prior to addition of other constituents. Cytochrome *b* was added with either a Hamilton syringe or a Pipetteman micro-pipetter directly to the stirred cuvette. All experimental data shown represent averages or typical results from at least two independent experiments on different preparations or from different days. Fluorescence quenching curves were transformed by first subtracting the baseline fluorescence, and then the absolute values of the fluorescence change were fit to a "one-phase exponential association" curve by non-linear regression using the equation $Y = Y_{\max}(1 - e^{-kx})$, where $Y_{\max}$ represents the fluorescence at saturation of the quenching effect. Fluorescence relaxation curves were also transformed by subtracting the baseline fluorescence, and the resulting values were fit to a "1-site binding (hyperbola)" curve from which the binding parameters of the anionic amphiphiles were calculated. All curve-fitting analyses were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com, Copyright (c) 1994-1999 by GraphPad Software. All rights reserved).

RET Distance Calculations.

Förster distance calculations were determined as described (78,79) assuming a K^2 value of 2/3, and a refractive index of 1.44. R_0 for the CCB-WGA probe was calculated at 47 Å allowing a useful range of approximately 25 to 90 Å for distance estimates between the donor and acceptor molecules. The quantum yield of the CCB-WGA molecule was determined by the method outlined in Parker and Rees (80) using quinine sulfate in 0.1N H₂SO₄ as a reference standard, quantitated using $\epsilon_{346} = 1.09 \times 10^4$, with a quantum yield = 0.51 ± 0.03 if $A_{346} < 0.02$ AU ($< 1 \mu\text{M}$) (81). Since the CCB labeling sites on the WGA are unknown, we assumed a spherical shape with a diameter of 50 Å, corresponding to an average axial dimension of the WGA molecule as determined from x-ray crystal coordinates (82,83). Due to the intrinsic planar symmetry of the heme molecule, the orientation relative to the donor dipole imposes at most a 20% error in any distance approximations (84).

Results

Resonance Energy Transfer Between Trisulfopyrenyl-labeled Wheat Germ Agglutinin and the Heme(s) of Cytochrome *b*.

A fluorescent conjugate of WGA, CCB-WGA, was used as a nonperturbing, extrinsic donor molecule for RET to the intrinsic heme ligands of cytochrome *b*. This

system was used to investigate whether allosteric effects are induced in cytochrome *b* by interactions with the cell-free oxidase activators SDS, LDS, and AA. The efficiency of the energy transfer, or in this instance, the degree of steady-state fluorescence quenching, allowed estimation of the average radial distance separating the donor-acceptor pairs based on Förster theory (78) (see Experimental Procedures). Figure 3.1 shows the spectral overlap between the normalized CCB-WGA fluorescence emission spectrum (λ_{ex} = 376 nm, solid line) and the Soret absorbance profile of the oxidized heme prosthetic groups of cytochrome *b* (dashed line). The spectrally-matched properties of the donor-acceptor pair satisfy the criteria required for RET (78) (for reviews, see Refs. 79,85,86) characterized by an R_0 value of 47 Å representing the distance at which 50% of the donor fluorescence would be quenched by the acceptor.

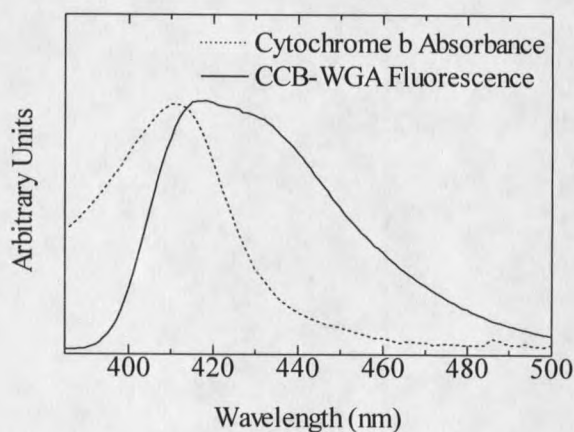


Figure 3.1. Overlap of Cascade Blue[®] fluorescence and cytochrome *b* absorbance spectra. Normalized oxidized Soret absorbance spectrum of cytochrome *b* (dotted line) in buffer A with λ_{max} at 414 nm. Emission spectrum of Cascade Blue[®] dye

when covalently bound to wheat germ agglutinin (CCB-WGA)(solid line) in buffer A, with $\lambda_{\text{ex}} = 376 \text{ nm}$ and λ_{em} maximum at 418 nm.

Addition of partially-purified cytochrome *b* ($\sim 15 \text{ nmoles heme}$) in a single aliquot to Buffer A containing 20 nM CCB-WGA conjugate, resulted in partial quenching of the CCB fluorescence (Figure 3.2 A). Titration of solutions containing CCB-WGA with aliquots of cytochrome *b* (Figure 3.2 B) caused a saturable, concentration-dependent quenching of up to $53.9 \pm 1.8\%$ ($n=4$) of the initial fluorescence. This value was somewhat variable ($\sim 20\%$) among blood donors and was inversely dependent on the age of the extract.

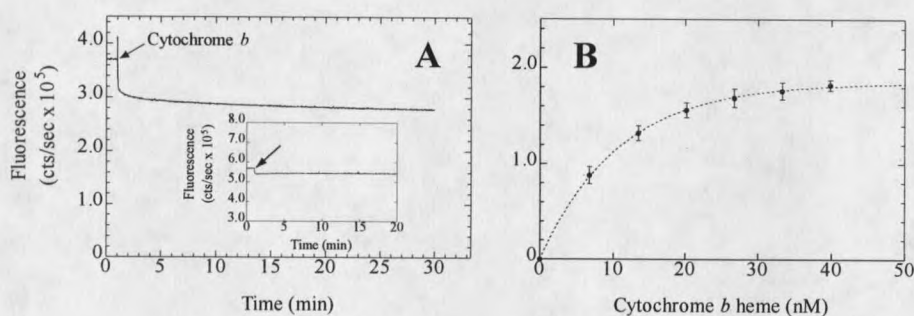


Figure 3.2. Quenching of CCB-WGA fluorescence by addition of cytochrome *b*.

(A) The effect of addition of cytochrome *b* ($\sim 15 \text{ nM heme}$) on the fluorescence of 20 nM CCB-WGA in buffer A, ($\lambda_{\text{ex}} = 376 \text{ nm}$, $\lambda_{\text{em}} = 420 \text{ nm}$). Time 0 to 1 min is the initial fluorescence in the absence of cytochrome *b*. At 1 min, partially-purified cytochrome *b* is added to a stirred cuvette and the fluorescence is monitored for a total of 30 min.

(Inset) A control consisting of cytochrome *b* added to free CCB dye. Time 0 to 1 min is the initial fluorescence of 20 nM CCB. At 1 min a single 20 μ L aliquot of cytochrome *b* (0.5 μ M heme) is added (final concentration of $\sim$ 20 nM) to the cuvette. The fluorescence is decreased by $\sim$ 4%, consistent with the dilution effect from the change in cuvette volume. (B) Concentration dependence of fluorescence quenching of 20 nM CCB-WGA by cytochrome *b*. Single 2 μ L aliquots of partially-purified cytochrome *b* (1.7 μ M heme) were added to a stirred cuvette containing 20 nM CCB-WGA. Following each addition of cytochrome *b*, the effects were allowed to equilibrate and the fluorescence was measured. These data were transformed by subtracting the baseline fluorescence and then plotting ($\circ$) the resultant absolute values $\pm$ SEM ($n = 4$). The transformed values were fit to a curve ($\cdots$) as described in the Materials and Methods section. At saturation concentrations of cytochrome *b*, the CCB-WGA fluorescence would be quenched a maximum of $53.9 \pm 1.8\%$, corresponding to an average heme to CCB moiety distance of 45.8 ± 0.5 Å.

Based on Förster calculations, this degree of quenching is consistent with an average CCB to heme radial distance of 45.8 ± 0.5 Å. Reverse titration of cytochrome *b* with CCB-WGA produced an analogous effect (not shown). Experimental concentrations of both the CCB-WGA and the cytochrome *b* heme were 20 to 40 nM, well below the level where any significant inner-filter effects would occur (for $\lambda_{\text{ex}} = 376$ nm, $A_{376} < 0.001$ AU for both CCB and cytochrome *b* heme) (79). Also, addition of 20

nM cytochrome *b* heme to solutions containing an equimolar amount of CCB-ethanolamine conjugate (Figure 3.2 A, inset) caused only a slight reduction in the fluorescence intensity, reflecting only dilution effects from cytochrome *b* addition. This control result indicated that any inner-filter effects were inconsequential to the observed fluorescence quenching and that the charged trisulfonic acid moieties of the CCB molecule did not promote non-specific ionic interactions between the molecules. Addition of equimolar concentrations of CCB-WGA to cytochrome *b* had no effect on the heme absorbance spectrum (not shown) indicating that formation of the CCB-WGA-cytochrome *b* complex did not perturb the heme environment. Together, these data suggest that the fluorescence quenching effect was due to resonance energy transfer between the donor-acceptor pairs, similar to our previous work with cytochrome *c* (R. Taylor, unpublished).

The Interaction Between the CCB-WGA Conjugate and Cytochrome *b* is a Lectin-carbohydrate Specific Interaction.

Native WGA has been shown to have both high affinity and specificity for GlcNAc and its multimers, and to a lesser extent, N-acetyl-neuraminic acid (sialic acid, NeuNAc) (87,88). To confirm that the same ligand specificity was retained between CCB-WGA and cytochrome *b*, we examined the effects of GlcNAc or the dimeric form, N-N'-diacetylglucosamine (chitobiose), or NeuNAc in both competition (Figure 3.3) and inhibition assays (not shown).

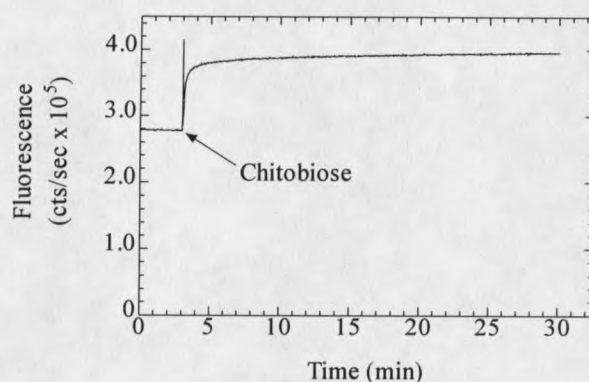


Figure 3.3. Chitobiose reverses CCB-WGA fluorescence quenching by cytochrome *b*. The effect of chitobiose on the fluorescence of 20 nM CCB-WGA in the presence of 40 nM cytochrome *b* heme. At 3 min, chitobiose dissolved in Buffer A is added in a single aliquot to a final concentration of 400 $\mu\text{g/mL}$, (942 μM) to the stirred cuvette. Rapid reversal of the fluorescence quenching is observed indicating competitive dissociation of the CCB-WGA, cytochrome *b* complex.

Addition of chitobiose to a final concentration of 400 $\mu\text{g/mL}$ (942 μM) to solutions containing 20 nM CCB-WGA previously complexed with equimolar cytochrome *b* heme, resulted in a rapid, total reversal of the fluorescence quenching (Figure 3.3). Complete inhibition of quenching was also observed when chitobiose was added to CCB-WGA prior to cytochrome *b* (not shown). In contrast, concentrations of GlcNAc or NeuNAc, up to 1 mM were ineffective in either reversal or inhibition of the quenching of the CCB-WGA fluorescence by the cytochrome *b*. Moreover, addition of either ligand to the CCB-WGA alone had no effect on the fluorescence (not shown).

These observations reflect the differences in the IC_{50} concentrations for chitobiose (0.15 mM), GlcNAc (12.5 mM), NeuNAc and sialic acid (25 mM) for inhibition of agglutinating activity by WGA (89). Since cytochrome *b* has a high GlcNAc content (71), these results also suggest that formation of the CCB-WGA cytochrome *b* complex and subsequent quenching is due to a lectin-carbohydrate interaction between the CCB-WGA and a multimeric GlcNAc on gp91^{phox}.

Reversal of quenching by addition of chitobiose was accompanied by an ~ 10% increase in the fluorescence beyond the initial amount prior to quenching (Figure 3). The increase was attributed to relaxation of a small amount of *intramolecular* quenching in the conjugated WGA (4.6 CCB per WGA dimer) upon its binding to complex sugars based on experiments utilizing other glycoproteins and proteinase K-digested cytochrome *b* (T. Foubert, unpublished observations). Together, these experiments suggest that the amount of *intermolecular* quenching of CCB-WGA fluorescence by cytochrome *b*, described above, might be a slight underestimate.

Fluorescence Quenching of CCB-WGA by Cytochrome *b* is Relaxed by SDS, LDS, and AA.

Most cell-free NADPH oxidase assays require addition of either SDS or LDS to initiate activity (90 -93). AA is another potent activator of O_2^- production and proton channel conductance by the NADPH oxidase when introduced either exogenously to intact phagocytes (68,94-96), or in cell-free systems (72), where it has been shown to

interact directly with cytochrome *b* (60). We thus exploited our system to investigate whether interactions between the activators and cytochrome *b* might induce structural changes in the cytochrome indicative of changes in the RET efficiency. Addition of aliquots of LDS (Figure 3.4 A, arrows), SDS (not shown), or AA (Figure 3.5 A, arrows) to CCB-WGA previously quenched by cytochrome *b* resulted in a concentration-dependent relaxation of the fluorescence quenching, with the effect approaching saturation at concentrations consistent with maximal cell-free oxidase system activity.

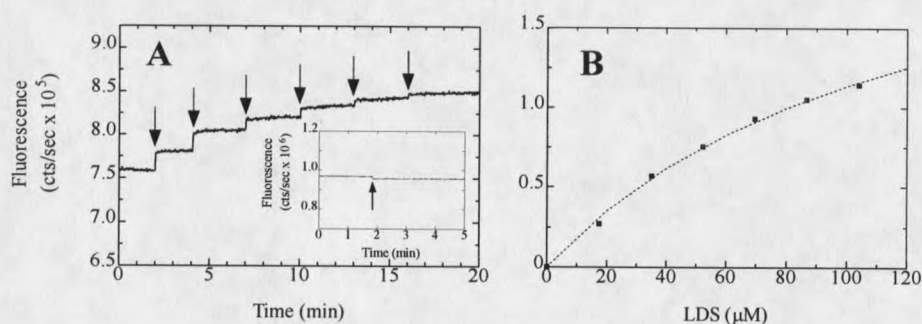


Figure 3.4. LDS relaxes CCB-WGA fluorescence quenching by cytochrome *b*.

The effect of addition of LDS to 20 nM CCB-WGA in Buffer A previously quenched by addition of 40 nM cytochrome *b* heme. **A)** Time 0-2 min is the cytochrome *b*-quenched CCB-WGA fluorescence. Beginning at 2 min, successive 1 μ L aliquots (arrows) of 10 mM LDS (~ 17.5 μ M final concentration) were added to the stirred cuvette to a final concentration of ~ 100 μ M. **(Inset)** Addition of a single aliquot of LDS (arrow) to a final concentration of ~ 120 μ M to CCB-WGA alone in Buffer A has no measurable effect on the probe fluorescence. **B)** The fluorescence intensity was measured immediately before

each successive addition of LDS from panel A. These data were transformed by subtracting the baseline fluorescence, and the values were plotted (■). The resulting plot was fit to a 1-site binding isotherm as described in the Materials and Methods section. The K_d for LDS was calculated to be $129.2 \pm 17.4 \mu\text{M}$ ($R^2 = 0.9977$), and extrapolation to the saturating concentration of LDS indicates that the fluorescence quenching would be completely relaxed.

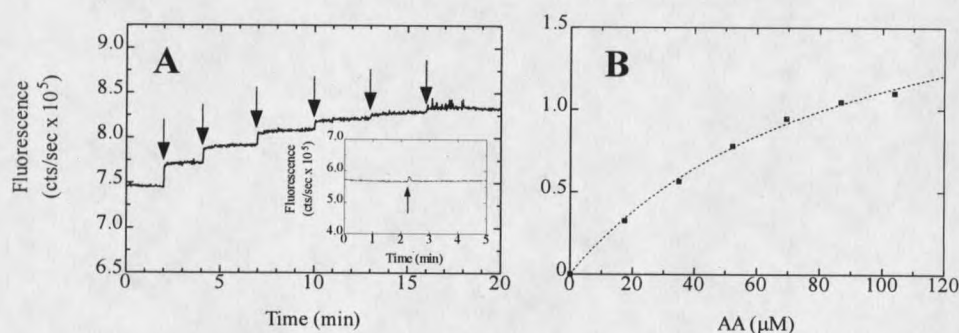


Figure 3.5. AA relaxes fluorescence quenching of CCB-WGA by cytochrome *b*. The effect of addition of AA to 20 nM CCB-WGA in buffer A previously quenched by addition of 40 nM cytochrome *b* heme. **A)** Time 0-2 min is the cytochrome *b*-quenched CCB-WGA fluorescence. Beginning at 2 min, successive 1 μL aliquots (arrows) of 10 mM AA ($\sim 17.5 \mu\text{M}$ final concentration) were added to the stirred cuvette to a final concentration of $\sim 100 \mu\text{M}$. **(Inset)** Addition of a single aliquot of AA (arrow) to a final concentration $\sim 300 \mu\text{M}$ to CCB-WGA alone in Buffer A has no measurable effect on the probe fluorescence. **B)** Fluorescence intensity measurements made immediately before each successive addition of AA were transformed by subtracting the baseline

fluorescence, and the values were plotted ($\square$). The resulting plot was fit to a 1-site binding isotherm ($\cdots$) as described in the Materials and Methods section. The K_d for AA was calculated at $93.1 \pm 11.5 \mu\text{M}$ ($R^2 = 0.9965$), and extrapolation to the saturating concentration of AA indicates that the fluorescence quenching would be completely relaxed.

The fluorescence values resulting from each successive addition of the activators were recorded after equilibrium was reached (Figures 3.4 B, 3.5 B) and fit to a binding isotherm modeling a 1-site binding interaction (see Experimental Procedures). Under these conditions, the K_d values for SDS, LDS, and AA were calculated to be 44.6 ± 4.8 , 129.2 ± 17.4 , and $93.1 \pm 11.5 \mu\text{M}$ respectively. Controls of SDS (not shown), LDS, or AA (Figures 3.4 A, 3.5 A, insets), added to CCB-WGA alone had no measurable effect on the probe fluorescence intensity. Additional experiments with SDS additions to 1.75 mM (SDS CMC = 1.33mM) to the CCB-WGA had no further effect on the probe fluorescence.

The methyl ester of arachidonate (AA-ME) is unable to elicit NADPH oxidase activity in either whole cells or the cell-free assay, and has been shown to competitively counteract AA-mediated oxidase activation (58,60,72,73). If the activator-induced relaxation effects were linked to oxidase activation, then addition of AA-ME to our RET system should cause dissimilar effects, and possibly be able to counteract those of AA.

Indeed, in contrast to AA, LDS, or SDS, addition of AA-ME to CCB-WGA previously quenched with cytochrome *b* caused a slight decrease in the steady-state fluorescence (Figure 3.6 A). Furthermore, when AA-ME was added after AA to the previously quenched CCB-WGA, the fluorescence relaxation effects from the AA were partially reversed (Figure 3.6 B).

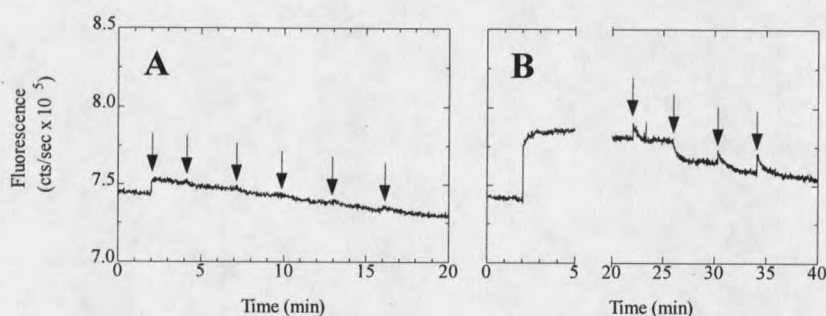


Figure 3.6. AA-ME has the opposite effect of AA on fluorescence quenching. The effects of addition of AA-ME on the fluorescence of CCB-WGA previously quenched by cytochrome *b*. **A)** Time 0-2 min is the fluorescence of 20 nM CCB-WGA previously quenched by exposure to 40 nM cytochrome *b* heme for 20 min. Beginning at 2 min, successive 1 μ L aliquots (arrows) of 10 mM AA-ME ($\sim 17.5 \mu$ M final concentration per aliquot) were added to the stirred cuvette to a final concentration of $\sim 100 \mu$ M. The fluorescence intensity is diminished slightly with each addition, an effect opposite of that observed with LDS, SDS, or AA. **B)** The reversal of the AA-induced relaxation effects by addition of AA-ME. Time 0-2 min is the fluorescence of 20 nM CCB-WGA previously quenched by exposure to 40 nM cytochrome *b* heme for 20 min. At 2 min a single 2 μ L aliquot of 10 mM AA ($\sim 35 \mu$ M final concentration) was added to

the stirred cuvette and allowed to equilibrate for 20 min. Beginning at 22 min, successive 2 μ L aliquots (arrows) of 10 mM AA-ME ($\sim 35 \mu\text{M}$ /per aliquot) were added to the stirred cuvette to a final concentration of $\sim 140 \mu\text{M}$. The AA-induced quenching effects are partially reversed by addition of AA-ME.

Relaxation of Fluorescence Quenching is Not Due to Dissociation of the Complex or Alterations in Heme Spectrum.

To ensure that the activator-induced fluorescence relaxation was not due to dissociation of the CCB-WGA complex, ELISA experiments were conducted. Cytochrome *b* was added to wells containing covalently-bound CCB-WGA (Figure 3.7) or WGA (not shown). Different concentrations of LDS or AA were added to the wells in buffer conditions analogous to the RET experiments and over the same time frame. The wells were then probed with $\alpha\text{-gp91}^{\text{phox}}$ mAb 54.1 (77).

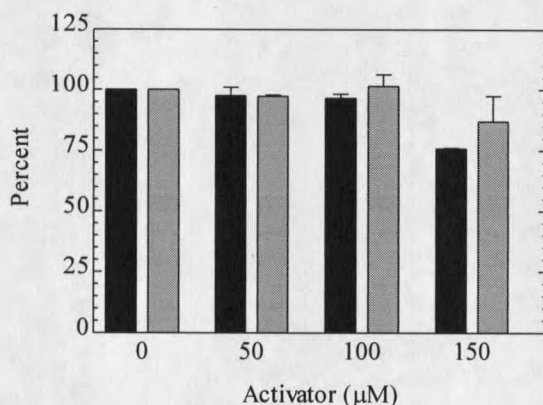


Figure 3.7. ELISA of cytochrome *b* bound to immobilized CCB-WGA after exposure to LDS and AA. Cytochrome *b* was bound to immobilized CCB-WGA on an

ELISA plate under essentially the same conditions and buffers as in the RET assays. The complexes were then exposed to varying concentrations of AA or LDS and probed with an α -cytochrome *b* mAb as described in Experimental Procedures. Shown are the relative amounts of cytochrome *b* bound to the immobilized CCB-WGA $\pm$ SD from two separate experiments with each concentration of either LDS (black bars) or AA (gray bars) done in triplicate. Data are expressed as a percent of control wells that contained only Buffer A. No significant change in the amount of cytochrome *b* bound by the CCB-WGA is observed at the 100 μ M experimental concentrations of either activator.

Activator concentrations of 150 μ M reduce the amount bound by approximately 25% for LDS and 14% for AA. Exposure of the WGA or CCB-WGA-cytochrome *b* complex to either LDS or AA at concentrations up to 100 μ M had little effect on the ELISA signal.

At activator concentrations between 100 and 150 μ M, both activators caused a partial loss of signal. Additionally, 120 μ M SDS had no effect on the ability of CCB-WGA to either bind immobilized fetuin, or to be eluted by chitobiose from this affinity matrix (not shown). These results indicate that the activator-induced quenching relaxation (Figures 3.4 and 3.5) was not due to dissociation of the CCB-WGA-cytochrome *b* complex.

Interactions between the activators and cytochrome *b* might also perturb the heme environment thereby affecting the Soret absorbance spectrum. The consequent changes in the spectral overlap between the donor-acceptor pair would contribute to the observed quenching relaxation. However, exposure of flavocytochrome *b* to 100 μ M SDS (Figure 3.8) or AA (not shown) had no effect on the Soret absorbance spectrum over a twenty

minute period in buffer conditions identical to the RET experiments. In contrast, at SDS or AA concentrations greater than $\sim 160 \mu\text{M}$, the Soret absorbance maximum was blue-shifted from 414 nm to 408 nm, concomitant with a reduction in the overall absorbance, and a loss of the 558 nm α -band following dithionite reduction (not shown). These observations suggested that activator concentrations greater than $\sim 160 \mu\text{M}$ perturbed the native heme environment probably by non-specific detergent-like interactions. These results correlated well with previous studies that showed maximum oxidase activity occurring at approximately 100-120 μM SDS or AA with higher concentrations corresponding to a sharp decline (72,90).

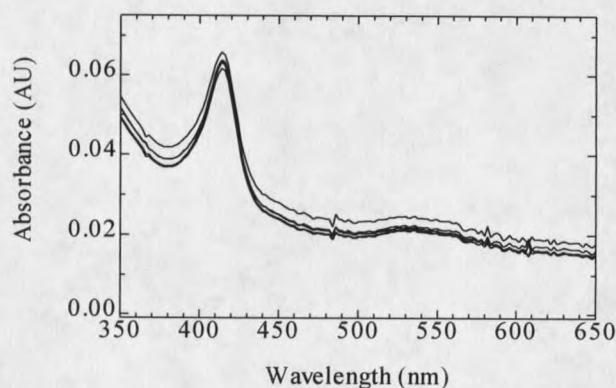


Figure 3.8. Stability of cytochrome *b* absorbance spectrum in the presence of 100 μM SDS. Absorbance spectrum of 0.5 μM cytochrome *b* heme in 200 μL Buffer A monitored during addition of 5 aliquots of 2 mM SDS over a 20 min time course to a final concentration of 100 μM . SDS at this concentration has no measurable effect on the Soret absorbance spectrum.

Discussion

Our results suggest that functionally-relevant structural rearrangements occur within cytochrome *b* concurrent with exposure to activators at concentrations known to activate the NADPH oxidase in cell-free systems. These results derive from a novel RET system that exploits the high lectin-carbohydrate affinity between CCB-WGA and the carbohydrate of gp91^{phox} to form a complex. Within this complex, the CCB moieties are positioned close enough to the intrinsic cytochrome *b* hemes to allow direct quantum mechanical transfer of electronic excitation energy. Consequently, up to ~ 55% of the CCB fluorescence is quenched, and subsequent additions of the anionic amphiphiles LDS, AA, or SDS to the complex relax the quenching implying a change in the spatial relationships between the donor-acceptor pairs.

Our system utilized Triton X-100-solubilized, partially-purified human neutrophil cytochrome *b* that was free of spectral interference from flavin or NADPH cofactors, both of which have intrinsic fluorescence and absorbance spectral overlaps with CCB. Detergent solubilization also circumvented the complexities of inter-cytochrome *b* RET due to aggregation or close proximity within the liposome, or orientation relative to the bilayer. By eliminating these confounding variables from our system, the RET effects that we observed could be interpreted within the context of a simplified donor-acceptor system consisting solely of CCB-WGA and monodisperse cytochrome *b*. At this degree of purity, the cytochrome *b* is capable of reconstituting defective respiratory burst activity in detergent extracts of neutrophil membranes obtained from patients with the autosomal

and X-linked forms of chronic granulomatous disease (97). In addition, it is able to bind the low molecular weight G protein rap1A in a phosphorylation-dependent manner (98,99). Most importantly, it displays both oxidized and reduced heme absorbance spectra identical to that of lipid-bound cytochrome *b* indicating a native heme environment. Thus, the detergent-solubilized cytochrome *b* used for these experiments represents a reasonable model of membrane-bound flavocytochrome *b*.

The complete relaxation of quenching at the calculated saturating concentrations of LDS, SDS, or AA to this RET system corresponds to an apparent displacement between the CCB-WGA and the flavocytochrome *b* hemes of roughly 30-40 Å based on Förster distance calculations (see Experimental Procedures). Since these effects appear to occur without perturbation of the heme spectrum, they probably represent a combination of structural rearrangements between inter- or intra- cytochrome *b* domains and reorientation of the heme dipoles (22,100) relative to the CCB donors. Such combinations that would yield results consistent with our RET data have been measured in other systems (101,102).

These changes could also be due to the activators interacting with domains of the protein that are not directly linked to oxidase or proton transport activity. However, the inability of AA-ME (Figure 3.6) to induce effects similar to AA would argue against this premise as it has been shown to act as an antagonist of AA in cell-free assays (60). Also, the activator-induced effects that we observe (Figures 3.4 and 3.5) approach saturation at concentrations consistent with those shown to elicit maximal activity in cell-free oxidase assays. This saturable behavior further suggests a specific binding event, thus

corroborating previous work (60). The analogous effects imparted by the different activators on our RET system also correlates well with their biochemical effects within the context of oxidase activation.

These significant effects may represent changes in cytochrome *b* structure that are linked to oxidase activity or proton transfer. The rationale for this proposition is based on the research of Beratan, Gray, and coworkers who calculated that small displacements on the order of 1-2 Å between aligned heme and aromatic protein side chains could result in orders of magnitude differences in electron tunneling probabilities through alpha helical regions of heme-bearing proteins (53-56,103-105). There are several examples of electron transferase proteins, such as rat liver cytochrome P450 (106), *T. pantotropha* cytochrome *cd*₁ nitrite reductase (103), cytochrome *c*₅₅₃ of *Desulfovibrio vulgaris* (107), mammalian cytochrome *c*/cytochrome *c* oxidase (84,108), and *Sulfolobus acidocaldrius* cytochrome *b*_{558/566} (102) that provide experimental support for this theory. Structural changes that occur in these proteins during the redox cycle represent a common mechanism for transducing mechanical motion into a means for control of transmembrane electron flow. The activator-mediated effects that we observed suggest that such a mechanism might also be functioning in cytochrome *b*.

Direct interactions between the activators and flavocytochrome *b* is supported by several studies. AA was shown to bind directly to cytochrome *b* (60), thereby affecting the binding affinities of oxygen and NADPH, and also affecting the accessibility of arsenic and cyanide derivatives to the cytochrome *b* hemes (59). Also, limited amounts of activator-induced superoxide production were achieved using purified, relipidated

flavocytochrome *b* in the absence of cytosolic proteins (47,60,109).

AA-mediated oxidase and proton translocation activity have both been shown to incorporate changes in heme ligation from a low spin, hexacoordinated, to a high spin, pentacoordinated state (22,58,103,110). Our results might therefore be a measure of this ligand switching, where positioning the heme geometry halfway between that of the ferri- and ferrocytochrome state (52,105,108) is in agreement with recently proposed intermediate states of NADPH oxidase activation (61). Another manifestation of these structural changes might be to promote oxygen binding directly to a cytochrome *b* heme (58), or to coordinate domain interactions at a remote site on the extracellular aspect of the protein (111).

Previous work has demonstrated that p47^{phox} (36,37,112) and possibly p67^{phox} (113,114) undergo activator-induced conformational changes during oxidase assembly. A limited amount of oxidase activity was also achieved using C-terminally truncated forms of p47^{phox} and p67^{phox} to activate a cell-free oxidase system in the absence of an anionic activator (113,114). These observations suggest that the activators interact with the cytosolic subunits to expose regions that can then interact with cytochrome *b* to partly control oxidase activation. These studies in combination with the present work suggest that complex relationships between the amphiphiles and protein components of the oxidase may exist that are integrated by cytochrome *b* during oxidase activation.

Activation of phagocytes temporally parallels modifications in their lipid content *in vivo*. Specifically, AA has been shown to be liberated *in situ* from the phagocyte membranes following receptor-mediated activation suggesting it may mediate both

oxidase (95,115-118) and proton channel activity (67,69) *in vivo*. AA appears to be liberated from phospholipids by the action of cytosolic phospholipase A₂ (cPLA₂) (67,119) under the control of a MAP kinase (41,120). A human myeloid cell line deficient in cPLA₂ was unable to activate the oxidase in response to various stimuli until supplemented with exogenous AA (121), and inhibitors of cPLA₂ were also shown to inhibit the oxidative burst (41).

Synthetic analogues of endogenous neutrophil lipids other than AA have been shown to either synergize with SDS and AA, or act alone as activators in cell-free oxidase systems (4,115,122-126). Preliminary results from testing certain lipid effectors on our system (T. Foubert, L. McPhail, A. Jesaitis, unpublished), chosen based on their ability to elicit superoxide generation *in vitro* (124), showed that certain phosphatidic acids but not diacylglycerols caused effects similar to SDS, LDS, and AA. Thus, LDS, SDS, AA, and possibly specific phosphatidic acids may define the requirements for effector action within our system; an anionic head group and either an unsaturated, or restricted length saturated fatty acid component, similar to results from previous studies (72).

Our work establishes a foundation for further investigation of the link between changes in the geometry of the flavocytochrome *b* and regulation of transmembrane electron and proton conduction. It also provides an assay for directly testing the induction of functionally-relevant structural changes in cytochrome *b* arising from interactions with specific compounds or possibly, the oxidase subunits. In preliminary studies (T. Foubert, A. Jesaitis, unpublished), we have observed that two CCB-labeled

monoclonal antibodies, mAb 7D5 (127,128) and mAb 44.1 (77,129) exhibit similar quenching from their respective extracellular and intracellular binding sites on detergent-solubilized cytochrome *b*. Moreover, on membrane-bound, fully functional flavocytochrome *b*, analogous relaxation effects have been observed for mAb 44.1 following addition of AA, SDS, and LDS. Accordingly, addition of AA-ME caused an opposite effect.

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CHAPTER 4

NADPH OXIDASE ACTIVATORS INDUCE STRUCTURAL CHANGES IN FLAVOCYTOCHROME *b*. FLUORESCENT CONJUGATES OF WHEAT GERM AGGLUTININ AND ANTI-FLAVOCYTOCHROME *b* MONOCLONAL ANTIBODIES AS EXTRINSIC DONORS FOR RESONANCE ENERGY TRANSFER.Introduction

Our previous work, discussed in chapter 3, utilized wheat germ agglutinin (WGA), conjugated with the acetyl azide derivative of the trisodium salt of 1,3,Trisulfo-8-pyrenyloxyacethydrazide, or Cascade Blue[®] acetyl azide (CCB) (1) as an extrinsic fluorescence donor for resonance energy transfer (RET) to the endogenous hemes of flavocytochrome *b*. Using this system, we demonstrated that certain NADPH oxidase activators (anionic detergents lithium- or sodium dodecyl sulfate (LDS, SDS) or the endogenous neutrophil phospholipid arachidonic acid (AA)) interact directly with detergent-solubilized flavocytochrome *b* to effect changes in the molecular geometry of the molecule. The magnitude of these effects was similar between the activators, approaching saturation at concentrations that correlated well with maximal oxidase activity in cell-free superoxide assays. Furthermore, the structurally similar oxidase antagonist, arachidonate methyl ester (AA-ME), induced effects that were opposite of those caused by SDS, LDS and AA suggesting chemical specificity. These observations supported our hypothesis that changes in the molecular geometry of flavocytochrome *b* are an integral component of NADPH oxidase activation.

In the first sections of this chapter, we describe exploitation of the CCB-WGA

RET system for examination of a subgroup of synthetic analogues of endogenous neutrophil lipids from a larger panel that had been assessed previously for their capacity to induce *in vitro* oxidase activity by Qualliotine-Mann *et al.* (2). This model system provided a framework to explore whether specific lipids might affect the molecular geometry of flavocytochrome *b* similar to SDS, LDS, and AA. If we could establish a correlation between their ability to induce oxidase activity and the extent of their effects on the structure of flavocytochrome *b*, our original hypothesis would be strengthened considerably. To this end, we tested the following synthetic phosphatidic acids (PA) and diacylglycerols (DAG) for effector action in our RET system: 1,2-distearoyl-sn-glycero-3-phosphate [1,2-(18:0)-PA], 1,2-dioleoyl-sn-glycero-3-phosphate [1,2-(18:1)-PA], 1,2-didecanoyl-sn-glycero-3-phosphate [1,2-(10:0)-PA] (a.k.a. dicapryl-PA), 1,2-dioctanoyl-sn-glycerol [1,2-(8:0)-DAG], and 1-oleoyl-2-acetoyleoyl-sn-glycerol [1-(18:1),2-(2:0)-DAG]. Within this group, the only lipids that induced fluorescence quenching relaxation effects similar to SDS, LDS, and AA were among the phosphatidic acids. The most dramatic effects were incurred by 1,2-didecanoyl-sn-glycero-3-phosphate, while the longer acyl chain phosphatidic acids showed a lesser degree of fluorescence relaxation. These preliminary results reflect the trends established by Qualliotine-Mann *et al.*, where phosphatidic acids with short (8 and 10 carbon) acyl chains were shown to elicit the highest oxidase activity, and increasing acyl chain length corresponded to decreasing activity. Our preliminary data suggest that effector action within this RET system minimally requires an anionic head group, and either a highly conjugated or restricted length acyl chain.

The last sections of this chapter explore the utility of previously characterized fluorescently-labeled mAbs as extrinsic donors for heme-mediated RET quenching experiments. The ability of a monoclonal antibody (mAb) to complex with its target epitope with such a high degree of specificity provides a unique interaction that can be exploited for RET studies. With exception to the V_H and V_L regions, X-ray crystallographic studies of IgG molecules have revealed conserved domain structures, including localization of the NH_2 -termini of both heavy and light chains within the distal portion of the Fab, outside of the antigen binding regions (3-5). Labeling schemes have been devised that exploit the amine-reactive functional groups on fluorescent dyes to preferentially target these amino termini (6,7) thus positioning the majority of the fluorescent moieties within roughly 20-30 Å of the antigen contact region.

Random-peptide phage display mapping of several α -flavocytochrome *b* mAbs, produced by our laboratory and others, has provided detailed information of the target epitope regions and proved invaluable for elucidating topographical features of flavocytochrome *b* (8-13). Labeling of these antibodies with spectrally-matched fluorescent dyes provides a system where RET spectroscopy can be utilized to examine the spatial relationships between separate epitope regions, or between individual epitopes and the intrinsic hemes of flavocytochrome *b*. Information derived from this approach should contribute to construction of a low resolution model of the tertiary and quaternary arrangement of the protein. Similar to our CCB-WGA system, the CCB-mAb system can also be used to monitor flavocytochrome *b* for structural changes that temporally parallel oxidase activity. Moreover, the singular epitope specificity of the mAbs provides a

means to distinguish between global and domain-specific structural changes.

Collectively, the information acquired from such experiments will assist in determining whether the changes in flavocytochrome *b* structure represent an allosteric control mechanism that is an integral step in oxidase or proton channel activation.

For these experiments, we utilized two CCB-labeled monoclonal antibodies, mAb 7D5 (8,14) and mAb 44.1 (10). Our preliminary results show that they exhibit quenching similar to CCB-WGA from their respective extracellular and intracellular binding sites on detergent-solubilized cytochrome *b*. Moreover, on membrane-bound, fully functional flavocytochrome *b*, relaxation effects analogous to the detergent solubilized CCB-WGA system have been observed for mAb 44.1 following addition of AA. Also in parallel with the detergent solubilized CCB-WGA RET system, addition of AA-ME caused an opposite effect.

Abbreviations

The abbreviations used are: AA, arachidonic acid, arachidonate; AA-ME, arachidonate methyl ester; CCB, Cascade Blue[®]; CGD, chronic granulomatous disease; CMC, critical micelle concentration; CNBr, cyanogen bromide; DDM, dodecyl maltoside; DAG, diacylglycerol; mAb, monoclonal antibody; GlcNAc, N-acetylglucosamine; NeuNAc, sialic acid, N-acetyl-neuraminic acid; NMWCO, nominal molecular weight cutoff; O₂⁻, superoxide anion; OG, octyl β-glucopyranoside, octylglucoside; PA, phosphatidic acid; PAGE, polyacrylamide gel electrophoresis; PAO,

phenyl arsine oxide; *phox*, phagocyte oxidase; RET, resonance energy transfer; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; 1,2-(18:0)-PA, 1,2-distearoyl-sn-glycero-3-phosphate; 1,2-(18:1)-PA, 1,2-dioleoyl-sn-glycero-3-phosphate; 1,2-(10:0)-PA, 1,2-didecanoyl-sn-glycero-3-phosphate, dicapryl-PA; 1,2-(8:0)-DAG, 1,2-dioctanoyl-sn-glycerol; 1-(18:1),2-(2:0)-DAG, 1-oleoyl-2-acetoxy-sn-glycerol.

Experimental Procedures

Materials.

KCl, NaCl, EDTA, NaN₃, gelatin, [ethylenebis(oxyethylenenitrilo)]Tetra acetic acid (EGTA), syringe filters for filtering buffers for fluorescence work, (Whatman, 25 mm diameter, polyethersulfone membrane, 0.2 μ m pore size) were purchased from Fisher Scientific (Tustin, CA). N-N'-diacetylglucosamine (chitobiose), Heparin-Sepharose 4B beads, N-formyl-Met-Leu-Phe, dihydrocytochalasin B, Na₂ ATP, chymostatin, wheat germ agglutinin (WGA), diisopropylfluorophosphate (DFP), sodium dithionite, quinine sulfate, arachidonate or the methyl ester suspended in chloroform, bovine serum albumin (BSA), HANKS balanced salts, TRIZMA-HCl (Tris-HCL), 30% hydrogen peroxide, ABTS, lithium dodecyl sulfate (LDS), goat α -mouse IgG conjugated to HRP, and Triton X-100 detergent were from Sigma Chemical Co (St. Louis, MO). All synthetic lipids were purchased from Avanti Polar Lipids, Inc (Alabaster, AL), either as a sodium salt or dissolved in chloroform. [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)]

(HEPES) and ultrapure SDS were obtained from United States Biochemical (Cleveland, OH). N-octyl- β -D glucopyranoside (OG, octylglucoside), dithiothreitol (DTT, Cleland's reagent), and phenylmethylsulfonyl fluoride (PMSF) were from Calbiochem-Novabiochem Corp (La Jolla, CA). 6 mm outside diameter, cylindrical, UV-transparent microcuvettes were purchased from Sienco, Inc (Färgelanda, Sweden). CCB-WGA conjugate, or CCB acetyl azide, was purchased from Molecular Probes (Eugene, OR). The BCA protein assay, supplied in kit form, was supplied by Pierce, Inc (Iselin, NJ), and Gammabind® Plus Sepahrose® beads were purchased from Biotech AB (Uppsala, Sweden).

Buffers.

Buffer A consisted of 150 mM NaCl, 10 mM Na₂PO₄, 0.72 mM Triton X-100 (0.045%, or 3X CMC), pH 7.4 and 0.02% NaN₃, in deionized water, filtered through a 0.2 μ m pore size membrane filter. Membrane resuspension buffer, MRB(-), was made as per the method of Parkos, *et al.* (15), and consisted of 10 mM NaCl, 100 mM KCl, 10 mM HEPES, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4, in deionized water. MRB(+) was MRB(-) supplemented with 1 mM PMSF and 10 μ g/mL chymostatin immediately before use. 10 mM phosphate buffered saline (PBS) contained 10 mM NaH₂PO₄, 150 mM NaCl, 2 mM NaN₃, pH 7.4, in deionized water. Buffer C consisted of MRB(-) and 0.72 mM Triton X-100. HANKS balanced salt solution was purchased as a premeasured dry powder and mixed as per the manufacturers instructions. Relax(+) consisted of 10

mM NaCl, 100 mM KCl, 10 mM HEPES, 3.5 mM MgCl₂, and 1.0 mM ATP, pH 7.4.

Buffers used for ELISA experiments were: Wash Buffer (10 mM HEPES, 150 mM NaCl in distilled water, pH 7.4), Blocking Buffer (Wash Buffer supplemented with 0.05% Tween 20 and 1% BSA), and ABTS reagent (50 mM sodium citrate in distilled water, pH 4.2 supplemented just before use with ABTS and hydrogen peroxide to final concentrations of 455 μ M and 0.1% respectively). Gammabind® Elution Buffer contained 0.5 M acetic acid, adjusted to pH 3.0 with neat NH₄OH. Equilibration Buffer consisted of 1.0 M Tris HCl, adjusted to pH 9.0 with 5 N NaOH. HPLC Size Exclusion Column Buffer contained 50 mM NaH₂PO₄, 150 mM NaCl, 2 mM NaN₃, pH 7.4, supplemented with either 0.1% Triton X-100, 40mM OG, or 0.2% dodecyl maltoside (DDM). Assay Buffer used for cell-free oxidase assays consisted of 1mM EGTA, 1mM MgCl₂, 2mM NaN₃, in 46.8 mM NaH₂PO₄, 18.2 mM K₂HPO₄, pH 6.8.

Purification of Cytochrome *b*:

Isolation of human polymorphonuclear leukocytes (PMNs) and purification of cytochrome *b* was carried out as described by Parkos *et al.* (15), or by immunoaffinity purification (R. Taylor, *et al.*, unpublished). Cytochrome *b* heme quantitation prior to heparin purification was determined from the reduced *minus* oxidized spectrum at 558 nm using $\Delta\epsilon_{558} = 29.3 \text{ (mM cm)}^{-1}$ (16), blanked against control buffer, and reduced by addition of sodium dithionite mixed in deionized water immediately prior to use, to a final concentration of 10 mM. After heparin purification, cytochrome *b* heme

quantitation was carried out using $\epsilon_{414} = 130.8 \text{ (mM cm)}^{-1}$ (16) blanked against buffer.

The absorbance was measured on either a Hewlett Packard HP 8452A diode-array spectrophotometer or a Molecular Dynamics Spectra-Max 250, environmentally controlled, micro titer plate spectrophotometer. When necessary, the samples were sonicated using a Fisher 50 probe style Sonic Dismembrator, model XL2005.

CCB-WGA Probe.

The CCB-WGA probe was stored in the dark either in lyophilized form at -20°C with desiccant, or suspended at 1 mM concentration in 10 mM PBS, pH 7.4, aliquoted, stored at -20°C , and thawed immediately prior to use. Quantitation of CCB-WGA was accomplished using $\epsilon_{400} = 31,400 \text{ (cm M)}^{-1}$ (Molecular Probes) for the CCB, and the WGA content back-calculated using the labeling stoichiometry average of 4.6:1, CCB:WGA (information supplied by Molecular Probes). Immediately prior to use, the probe was diluted in Buffer A to a working concentration of 10 or 20 nM, based on WGA content, and filtered through a $0.2 \mu\text{m}$ pore size syringe filter to remove particulate contamination. To prevent moisture condensation on cuvettes, the working solution was brought to room temperature before use.

ELISA

Seventy five μL of a solution containing 6 μM WGA and 860 μM NAG in DPBS were added to the wells of a 96-assay ELISA plate and incubated 6 hours at 25° C. The wells were rinsed 3 times with DPBS and blocked by exposure to 300 μL per well of Blocking Buffer for 48 h at 4° C. Flavocytochrome *b* was purified to the heparin affinity step (above), and eluted using 2% OG without Triton X-100. The mixture was then diluted to a final OG concentration of 0.02% and 40 μL was added to each well and incubated for 6 h at 25° C (~ 8-10ng heme per well). The wells were then washed 3 times with DPBS containing 10 % Triton X-100, and the peptide-antibody mixture or antibody alone in Blocking Buffer was added to the wells and incubated overnight at 4° C. The plate was then washed three times with Blocking Buffer and 100 μL of goat α -mouse horse radish peroxidase conjugated IgG secondary antibody was added per well at a 1:1000 dilution in Blocking Buffer and incubated for 1 hour at room temperature. The plate was then rinsed 5 times with Wash Buffer and developed by adding 100 μL of ABTS reagent per well. Absorbance values were measured at 405 nm.

CCB-mAb Conjugation

Antibodies were covalently labeled under neutral pH conditions where isothiocyanates and acetyl azides remain preferentially reactive with amino groups (7),

and the NH_2 -terminal α -amino groups of the mouse IgG heavy and light chains are more reactive than most of the accessible ϵ -amino groups (6). The monoclonal IgG antibodies (mAb) 7D5 (8,14), 44.1 (10,13), and H7 (J. Burritt, unpublished) were isolated using a Gammabind® affinity matrix to remove albumin that was used as a stabilizing agent. ~ 1 mL of Gammabind® beads were added to a 4 mL fritted column, drained, and rinsed with 5 mL of Elution Buffer. The beads were then equilibrated by rinsing with 5 mL of 10 mM PBS, and the antibodies (2-4 mg) were added directly and mixed end over end overnight at 4° C. Columns were then drained and rinsed with 20 mL of 10 mM PBS followed by 10 mL of 10 mM PBS with 0.5 M NaCl, and equilibrated with 10 mL of 10 mM PBS. The antibodies were eluted by overlaying 5 mL pH 3 Elution Buffer onto the beads and collecting 500 μL fractions directly into a 400 μL volume of Equilibration Buffer. The TRIS from the equilibration buffer was removed by repeated dialysis against 10 mM PBS. The antibody containing solutions were then concentrated to ~ 1 mL final volume using a 30 kDa NMWCO centrifugal concentration device, and the final protein content of the individual antibodies was determined using the BCA method. A 50-fold molar excess of CCB acetyl azide dye from a 10 mM stock solution dissolved in methanol was added and mixed end over end for 48 h at 4° C. The reaction was terminated by addition of 200 μL of 1.5 M hydroxylamine, pH 8.5 and mixed end over end for 1 h at room temperature. Excess dye was removed by either extensive dialysis against 10 mM PBS or by gel filtration using disposable desalting columns (Biorad, # 22314) followed by extensive dialysis against 10 mM PBS. The protein content of the labeled antibodies was determined using the BCA method, and the CCB quantitated by

absorbance using $\epsilon_{400} = 31,400$ (information supplied by Molecular Probes). The final CCB:mAb labeling stoichiometry of the individual antibodies was determined to be 5.7:1, 5.3:1, and 3.6:1 for mAbs 44.1, 7D5, and H7 respectively. Labeled antibodies were stored wrapped in foil at 4° C for several months without loss of activity.

Lipids.

All lipid stocks were stored in the dark at -20° C, in air-tight containers that had been purged with argon. Working solutions from lipids dissolved in chloroform were prepared by transferring aliquots from the stock to a fresh tube where the chloroform was removed by evaporation in a stream of ultra high purity argon at room temperature. The condensate, and also lipid stocks supplied as sodium salts, were resuspended in deionized water by brief probe sonication, purged with argon, and kept on ice prior to use. All working solutions of lipids were prepared fresh the same day as used.

Cell-Free Oxidase Assay.

Plasma membrane-bound flavocytochrome *b* and cytosolic fractions were prepared using LPS-free reagents as described (17), with the following modification. A discontinuous 5-20% Percoll gradient was used to fractionate the subcellular constituents. Concentration dependence analysis was used to determine the optimal concentrations of plasma membranes, cytosol, and LDS (data not shown). Assays were performed in Costar

3370 96-well microtiter plates in Assay Buffer supplemented with 100 μM cytochrome *c*, 125 μM LDS, 10 μM FAD, 200 μM NADPH, 125 μM GTP γ S, 5×10^5 cell-equivalents of plasma membranes, and 2×10^5 cell equivalents of the cytosol in a total volume of 200 μL . Superoxide was measured as the superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c*, quantitated using $\Delta\epsilon_{550} = 21 \text{ (cm mM)}^{-1}$ (18) and blanked against identical wells containing 375 U/mL SOD.

Fluorescence Measurements.

All reagents containing CCB were kept in foil-wrapped containers and solutions containing cytochrome *b* or lipids were kept on ice prior to use. Experiments were conducted at ambient temperature in cylindrical 6 mm outside diameter 500 μL continuously-stirred UV transparent microcuvettes. Fluorescence measurements were recorded using a Photon Technologies Inc. Quanta-Master QM-1 steady state fluorometer with excitation monochromators set at 376 nm and the emission monochromator set at 418 nm for kinetic measurements. The slit width for both monochromators were set at 2 mm, corresponding to a 4 nm and 8 nm bandpass for the excitation and emission monochromators, respectively. All fluorescence spectra were corrected for the wavelength-dependent instrument response using the software functions provided by the manufacturer (Felix software ver 1.1, 1996 PTI, Inc). For all experiments the CCB-conjugated proteins in solution (10 or 20 nM) were introduced to a continuously-stirred cuvette, and the fluorescence was monitored for a short period prior to addition of other

reagents. Cytochrome *b* was added with either a Hamilton syringe or a Pipetteman micro-pipetter directly to the stirred cuvette. All curve-fitting analyses were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com, Copyright (c) 1994-1999 by GraphPad Software. All rights reserved).

Absorbance Measurements.

Quantitation of CCB was carried out using $\epsilon_{400} = 31,400 \text{ cm}^{-1} \text{ M}^{-1}$ (Molecular Probes). Protein quantitation, to determine labeling stoichiometry was conducted using the BCA method as described by Pierce, compared to bovine serum albumin standards. Quantitative absorbance measurements were made using either a Hewlett Packard HP 8452A diode-array spectrophotometer or a Molecular Dynamics Spectra-Max 250, environmentally controlled, micro titer plate spectrophotometer.

RET Distance Calculations.

Förster distance calculations were determined as described (19,20) assuming a K^2 value of 2/3, and a refractive index of 1.44. The quantum yields of the CCB-mAb conjugates were determined by the method outlined in Parker and Rees (21) using quinine sulfate in 0.1N H_2SO_4 as a reference standard, quantitated using $\epsilon_{346} = 1.09 \times 10^4$, with a quantum yield = 0.51 ± 0.03 if $A_{346} < 0.02 \text{ AU}$ ($< 1 \mu\text{M}$) (22). The quantum yields

of the mAbs were determined to be 0.50, 0.57, and 0.66, for mAb 44.1, 7D5, and H7 respectively. R_0 values calculated for the donor mAbs 44.1, 7D5, and H7 to heme acceptor are 53.6, 54.6, and 56.1 Å respectively, and the R_0 value for CCB-44.1 to dabsyl acceptor was calculated at 48.8 Å. These R_0 values provide a useful range of roughly 25 to 90 Å for distance estimates between the donors and acceptors.

Peptide Dabsylation.

Peptides Ac-PQVNRPI-CONH₂, NH₂-ATAGRFGGPQVNPI-CONH₂, and NH₂-ADNRPWGPGPYGP-CONH₂ were supplied by Macro Molecular Resources, Boulder, CO. 5 mg of the peptide NH₂-ATAGRFGGPQVRPI-CONH₂ was reacted with a 1:1 molar ratio of dabsyl chloride in 40 µL of dimethylformamide (DMF) for 1h at room temperature, with 4µL aliquots of triethanolamine (TEA) added every 15 minutes to maintain a basic pH. Peptides with a single dabsyl label were separated from other populations and free dabsyl by reverse-phase HPLC chromatography. Labeling stoichiometry was confirmed by MALDI-MS as described below, and also by comparing protein content (determined by BCA assay) to absorbance of the dabsyl group. The molar extinction for the dabsyl group in aqueous buffers was determined experimentally. The dabsylated peptide was dissolved in methanol and the concentration determined using $\epsilon_{438} = 31,000$ (information supplied by Sigma). An equivalent amount of dabsylated peptide was then dissolved in HPLC Size Exclusion Buffer, and the molar extinction coefficient was back calculated. λ_{max} shifted from 438 nm in methanol to 452

nm in aqueous solution, and ϵ_{452} was calculated to be 24,500. 3 species were identified in separate HPLC fractions with masses corresponding to 0, 1, and 2 dabsyl groups per peptide. Labeled peptides were then compared to non labeled peptides by ELISA competition assays as described above. Single dabsyl modification had no significant effect on peptide binding under the ELISA conditions.

HPLC Chromatography.

All samples were passed thru a 0.2 μ m pore size syringe filter prior to HPLC separation. All HPLC analyses were conducted using a Hitachi, LS-6200 HPLC with an F-1050 fluorescence detector connected in series with an L-7450A UV-Visible Diode Array Detector. Size exclusion chromatography was conducted using a Biorad Superdex 200 HR 10-30 column, maintained at 4° C, with a flow rate of 0.5 mL/min, equilibrated in HPLC Size Exclusion Column Buffer. Elution fractions were collected at 1 min intervals throughout the 1 h runs and placed immediately on ice. All flavocytochrome *b* containing fractions were supplemented with PMSF and chymostatin to final concentrations of 1 mM and 10 μ g/mL, respectively. Separation of dabsylated peptide species was conducted at room temperature using a Vydac reverse phase polymer column, 4.8 x 15 cm (cat # 259VHP5415). Samples were prepared by diluting 1:10 with distilled water containing 0.01% TFA, and eluted using 10-90% linear gradient of CH₃CN with 0.01% TFA in distilled water, at a flow rate of 1 mL/min.

Mass Spectrometry.

MALDI-TOF MS data were collected on a PerSeptive Biosystems/Vestec Voyager (PerSeptive Biosystems, Framingham, MA) benchtop time-of-flight (TOF) mass spectrometer. Samples were lyophilized, resuspended in a solution of 1:1 CH₃CN:distilled water containing 0.01% (w/w) TFA, and aliquots were mixed 1:1 with a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in a 90:10 water:ethanol solution. 1 μ L samples were spotted onto a MALDI-MS plate and allowed to air dry. Samples were then analyzed in linear mode with masses determined relative to a 2-point standard consisting of cytochrome *c* and matrix.

Preparation of Neutrophil Cytosol.

Human polymorphonuclear leukocytes were isolated as described by the method of Parkos *et al.* (15,23), suspended in ice cold HANKS buffer and DFP was added neat to a final concentration of 0.5 μ L / 1×10^8 cells/mL. Cells were then incubated on ice for 15 min with intermittent mixing, pelleted at 400 x g for 10 min at 4° C, and resuspended to a concentration of 1×10^8 cells/mL in 4° C Relax(+). Cells were then equilibrated to 450 psi with N₂, at 4° C for 20 min in a stirred vessel, and disrupted by nitrogen cavitation. The cavitate was collected at 4° C in a vessel containing 1/10 the precavitation volume of Relax(+) supplemented with 12.5 mM EGTA, 1mM DTT, 10mM PMSF, and 100 μ g/mL chymostatin. Nuclei and unbroken cells were pelleted by

centrifugation at 1000 x g for 5 min at 4° C. The supernatant fraction was collected, transferred to new centrifuge tube, and centrifuged at 144,000 x g for 30 min at 4° C. The supernatant fraction was then collected, dispensed into aliquots, and stored at -70° C. The fractions tested positive for their reconstitution ability in a cell-free superoxide assay as described (24)

Results

Specific Synthetic Phospholipids Induce Structural Changes in Flavocytochrome *b* Similar to SDS, LDS, and AA.

Using the CCB-WGA RET system described in chapter 3 of this dissertation, we tested a group of synthetic lipids for their ability to induce structural changes in flavocytochrome *b* similar to SDS, LDS, and AA. Our intent was to determine if a correlation existed between their efficacy in eliciting oxidase activity *in vitro*, and their ability to induce perturbations of flavocytochrome *b* structure. The lipids used for these preliminary studies were chosen on the basis of results from a previous study conducted by Qualliotine-Mann *et al.* (2) who used a neutrophil derived, broken-cell superoxide assay to test different combinations and concentrations of synthetic lipids for their ability initiate oxidase activity in the absence of AA or anionic detergents. They were able to identify a subgroup of lipids that showed substantial biochemical activity alone or could synergize with others, while others were inactive. The highest degree of oxidase activity

using only phosphatidic acids was achieved at $\sim 300 \mu\text{M}$ concentrations of 2-(8:0)-PA or 1,2-(10:0)-PA. The highest degree of synergistic activity, achieved in the presence of $10 \mu\text{M}$ PA, occurred at a concentration of $30 \mu\text{M}$ 1,2-(8:0)-DAG. Overall, lipids with short chain (e.g. 8-12 carbon) saturated acyl chains were more active than longer chains (e.g. 16-18 carbon) and longer chains retained some activity by inclusion of conjugated bonds.

We tested 3 of the synthetic PA analogues in our CCB-WGA RET system, 1,2-distearoyl-sn-glycero-3-phosphate [1,2-(18:0)-PA], 1,2-dioleoyl-sn-glycero-3-phosphate [1,2-(18:1)-PA], 1,2-didecanoyl-sn-glycero-3-phosphate [1,2-(10:0)-PA] (a.k.a. dicapryl-PA). Addition of the first of 2 separate aliquots of 1,2-(10:0)-PA, to a final concentration of $100 \mu\text{M}$, caused a dramatic relaxation of the fluorescence quenching by flavocytochrome *b* (Figure 4.1 A).

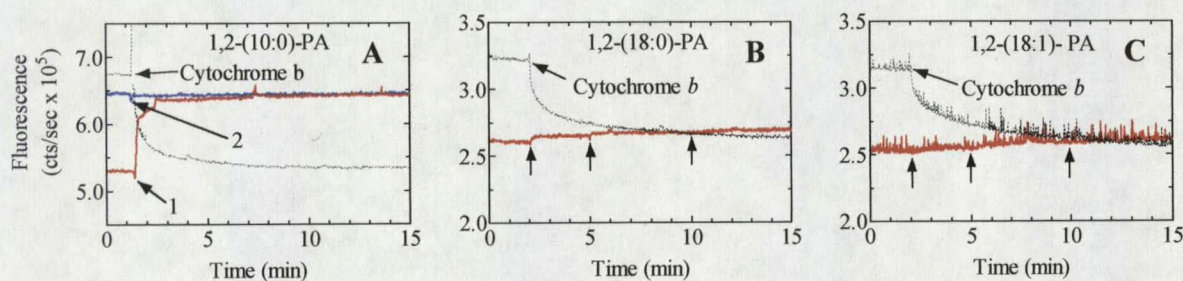


Figure 4.1. PA relaxes the CCB-WGA fluorescence quenching by flavocytochrome *b*. Panels show the fluorescence relaxation effects of individual synthetic phospholipids when added to a cuvette containing 20 nM CCB-WGA previously quenched by addition of Triton X-100 solubilized, partially-purified flavocytochrome *b*. A) A single $5 \mu\text{L}$ aliquot of flavocytochrome *b* is added at 2 min to

20 nM CCB-WGA in Buffer C (black line) and the fluorescence is monitored for the remainder of a 15 min run. The run is continued (red line) and at 2 min (arrow 1) a single aliquot of 1,2-(10:0)-PA is added yielding a final concentration of 100 μ M, and the effects are allowed to equilibrate for the remaining 15 min. The run is again continued (blue line) and a second identical aliquot is added. **B)** The fluorescence of 20 nM CCB-WGA is quenched the same as described for Panel A (black line). The run is continued (red line) and successive 5 μ L aliquots of 1,2-(18:0)-PA are added at the indicated time points (arrows) to a final concentration of 30 μ M while monitoring the fluorescence. **C)** The same experiment as described for Panel B but using 1,2-(18:1)-PA.

Addition of the second aliquot had no further effect indicating that the effects imparted by the phospholipid saturated at a concentration of 100 μ M or less. Addition of either 1,2-(18:0)-PA (Figure 4.1 B), or 1,2-(18:1)-PA (Figure 4.1 C) under identical conditions to final concentrations of 30 μ M also relaxed the fluorescence quenching, though to a lesser extent. In contrast, addition of either 1,2-(8:0)-DAG (Figure 4.2 A), or 1-(18:1),2-(2:0)-DAG (Figure 4.2 B) under the same conditions to final concentrations of 200 μ M failed to relax the quenched fluorescence at all. It is noteworthy that the PA that caused the most dramatic effects in our RET system also showed the highest *in vitro* biochemical activity in the report of Qualliotine-Mann *et al.* A well designed study conducted recently by Erickson *et al.* (25) tested a similar set of synthetic lipids for their ability to initiate oxidase activity *in vitro*. Their cell-free system also consisted of membrane and cytosolic fractions of neutrophil origin, but lacked the long incubation

periods as well as the other non physiological constituents of the previous study of Qualliotine-Mann *et al.* They were able to demonstrate PA-mediated oxidase activity comparable to SDS within a physiologic time frame (2-5 min) using both deoxycholate-solubilized and membrane-bound flavocytochrome *b*.

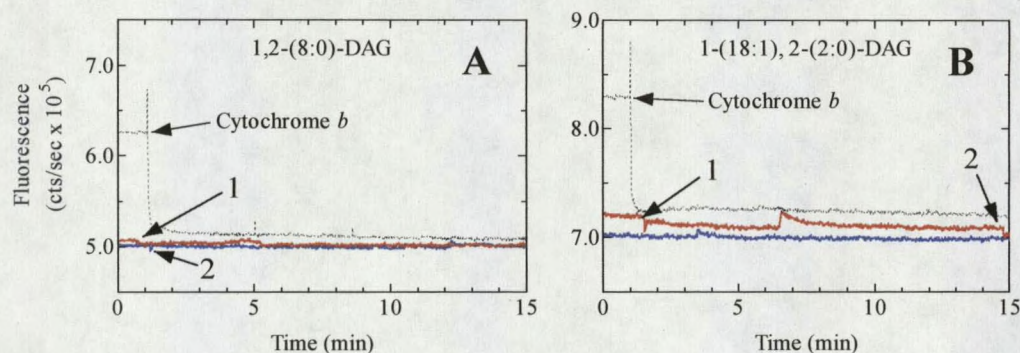


Figure 4.2. DAG does not relax the CCB-WGA fluorescence quenching by flavocytochrome *b*. **A)** A single 5 μ L aliquot of flavocytochrome *b* is added at 2 min to 20 nM CCB-WGA in Buffer C (black line) and the fluorescence is monitored for the remainder of a 15 min run. The run is restarted (red line) and at 1 min (arrow 1) a single aliquot of 1,2-(8:0)-DAG is added yielding a final concentration of 100 μ M, and the fluorescence relaxation effects are allowed to equilibrate for the remaining 15 min. The run is restarted (blue line) and a second identical aliquot is added. **B)** The fluorescence of 20 nM CCB-WGA is quenched the same as described for Panel A (black line). The run is continued (red line) and at 2 and 14 min, identical 5 μ L aliquots of 1-(18:1),2-(2:0)-DAG are added to a final concentration of 200 μ M. The run is continued (blue line) and the fluorescence is monitored for an additional 15 min.

More importantly, they were able to establish that effector action by DAG was dependent on its conversion to PA via a diacylglycerol kinase, though only a small percentage was converted. Further investigation revealed that suboptimal concentrations of 1,2-(8:0)-PA could synergize with 1,2-(8:0)-DAG to increase oxidase activity by unknown mechanisms, thus corroborating the earlier observations of Qualliotine-Mann *et al.* These observations could serve to explain the lack of effector action by the DAGs in our RET system in the absence of PA. Our RET data and both of the previous reports are consistent in the observations that PA with short acyl chains (e.g. 8 and 10 carbon) were the most effective activators, and activity diminished as a function of increasing chain length. Further RET studies with a full spectrum of synthetic lipids need to be conducted to identify which lipids interact with flavocytochrome *b* and to what extent they exert their effects.

CCB-labeled mAbs.

This section describes an extension of our previous work in investigation of the possible link between structural changes in flavocytochrome *b* and oxidase activation. Two α -flavocytochrome *b* monoclonal antibodies (mAbs), 44.1 (10,13) specific to the intracellular aspect of p22^{phox}, and 7D5 (8,14) to the extracellular aspect of gp91^{phox}, and a control of the same isotype, mAb H7, specific for hCAP-18 (26-29) were covalently labeled with the acetyl azide derivative of the trisodium salt of 1,3,Trisulfo-8-pyrenyloxyacethydrazide, or Cascade Blue[®] acetyl azide (CCB) (1). The CCB:mAb

labeling stoichiometries for mAbs 44.1, 7D5, and H7 were determined to be 5.7:1, 5.3:1, and 3.6:1, respectively. Under the conditions used for conjugation (see Experimental Procedures), ideal results would have yielded a 4:1 CCB:mAb labeling stoichiometry corresponding to a single label at the NH₂ termini of each heavy and light chain. SDS-PAGE separation of the individual CCB-mAbs followed by immunoblotting with α -CCB antibodies revealed a relatively equivalent distribution of CCB label between the heavy and light chains (Figure 4.3 top).

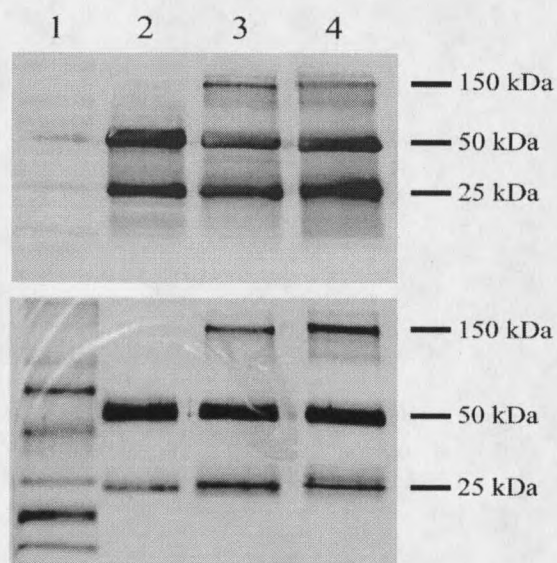


Figure 4.3. CCB label distribution between light and heavy chains of mAbs.

CCB-labeled mAbs were separated by SDS-PAGE, and either immunoblotted using α -CCB primary antibodies (top) or silver stained (bottom) as described in Experimental Procedures. For both panels, lanes 1-4 are molecular mass markers, CCB-44.1, CCB-

7D5, and CCB-H7, respectively. All 3 antibodies show a relatively consistent labeling distribution between heavy and light chains.

The labeled mAbs were then compared to non-labeled mAbs by ELISA to determine if incorporation of the trisulfonic acid moieties of the CCB molecule might affect their ability to bind their respective epitopes (Figure 4.4). These results indicated that conjugation had little effect on the binding capacity of these mAbs. Collectively, these results suggest that on average, the NH₂-termini of all 3 mAbs are probably labeled as well as additional sites that are likely located outside of the antigen binding regions of mAbs 44.1 and 7D5.

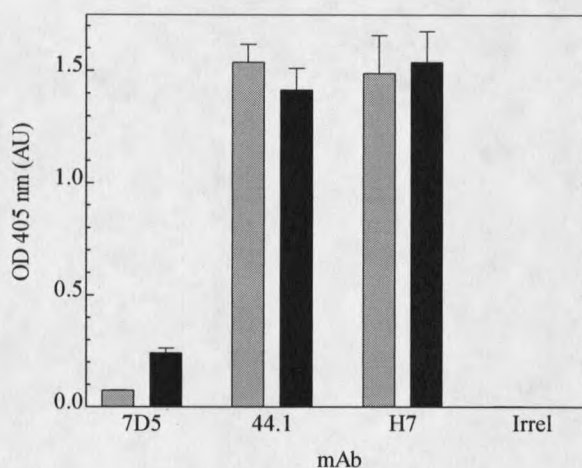


Figure 4.4. ELISA comparing CCB-labeled and unlabeled mAb binding to immobilized, detergent-solubilized flavocytochrome *b*. Heparin eluate was prepared from neutrophil membranes as described in Experimental Procedures. The CCB-labeled

mAbs (black bars) shown in Figure 1 were compared to unlabeled mAbs (gray bars) relative to an irrelevant mAb for their antigen binding capacity. CCB labeling did not significantly affect binding of mAbs 44.1 and H7, and CCB-labeled mAb 7D5 exhibited a slightly increased binding. Data shown are the average values $\pm$ SD from a single experiment with each mAb done in triplicate and are consistent with two separate experiments using different neutrophil membrane preps.

Fluorescence Quenching of CCB-labeled
Antibodies by Detergent-solubilized Flavocytochrome *b*.

We next investigated the ability of the CCB-labeled mAbs to complex with detergent-solubilized flavocytochrome *b* and quench the CCB fluorescence similar to our CCB-WGA system. Twenty μ L aliquots of immunoaffinity-purified flavocytochrome *b* (500 nM heme) solubilized in DDM HPLC column buffer were added to the individual CCB-mAbs while monitoring their steady-state fluorescence intensity (Figure 4.5). The fluorescence of 20 nM (protein) CCB-7D5 (Figure 4.5 A) is quenched a total of $\sim 32\%$ (dilution corrected value) at a heme concentration of ~ 54 nM. Addition of the third aliquot of flavocytochrome *b* decreased the fluorescence only by an amount slightly greater than the dilution effects incurred by the change in cuvette volume (compare to Figure 4.5 C) indicating that the fluorescence quenching effects were close to saturation at a molar ratio of roughly 3:1 flavocytochrome *b* heme:CCB-7D5 (protein). Although this amount of quenching is consistent with an average separation distance of 62 Å

between the CCB donors and the heme acceptors of flavocytochrome *b*, the saturation limit cannot be extrapolated by curve fitting due to an insufficient number of data points. The implied 62 Å separation distance, therefore, represents an upper limit on the average distance between the donors and acceptors.

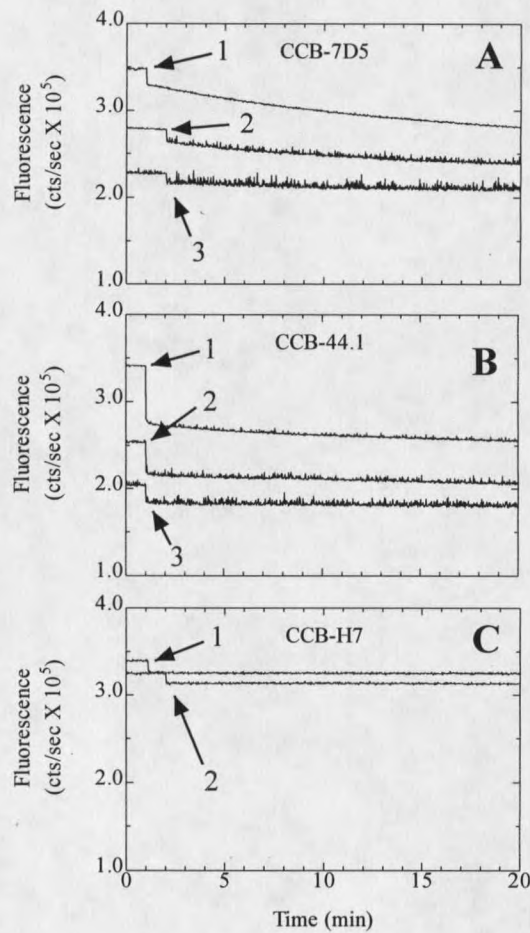


Figure 4.5. Fluorescence quenching of CCB-labeled mAbs by addition of detergent-solubilized, purified flavocytochrome *b*. 20 μ L aliquots of immuno-purified

flavocytochrome *b* (~ 500 nM heme) prepared as described in Experimental Procedures was added to cuvettes containing 20 nM (protein) CCB-mAbs suspended in DDM HPLC Buffer at the indicated time points (arrow 1), and the binding interaction was allowed to equilibrate for 20 min. This sequence was repeated 2 more times (arrows 2 and 3) for CCB-mAbs 7D5 (Panel A), and 44.1 (Panel B), and 1 more time for the control CCB-mAb H7 (Panel C). The fluorescence intensity of both α -flavocytochrome *b* CCB-mAbs is partially quenched by addition of flavocytochrome *b*, while the fluorescence intensity of the control CCB-H7 is diminished only by an amount consistent with the dilution effect caused by the change in cuvette volume.

Similar fluorescence quenching effects were observed with CCB-44.1 (Figure 4.5 B), but at the same ~ 54 nM heme concentration the CCB-44.1-flavocytochrome *b* interaction is less saturated than that of CCB-7D5. At this heme concentration, the CCB-44.1 fluorescence is quenched by 40%, suggesting an upper limit on the donor-acceptor separation distance of 57 Å. Addition of the same concentrations of flavocytochrome *b* to the negative control mAb CCB-H7 (Figure 4.5 C) did not reduce the fluorescence intensity beyond the dilution effects from the change in cuvette volume. Additional experiments will need to be conducted to determine the saturating concentrations of flavocytochrome *b* that result in maximal fluorescence quenching of each respective CCB-mAb.

Isolation of CCB-44.1 mAb Complexed With
Flavocytochrome *b* by HPLC Size Exclusion Chromatography.

We next attempted to isolate complexes consisting solely of monodisperse flavocytochrome *b* and CCB-44.1 to gain a more accurate measure of the fluorescence quenching by eliminating those populations of molecules that were not interacting. Partially purified, detergent-solubilized flavocytochrome *b* was incubated with the CCB-mAb 44.1 conjugate as described under Experimental Procedures to promote formation of mAb-flavocytochrome *b* complexes. The mixture was then subjected to HPLC size exclusion chromatography to separate the different populations of bound and unbound proteins (Figure 4.6).

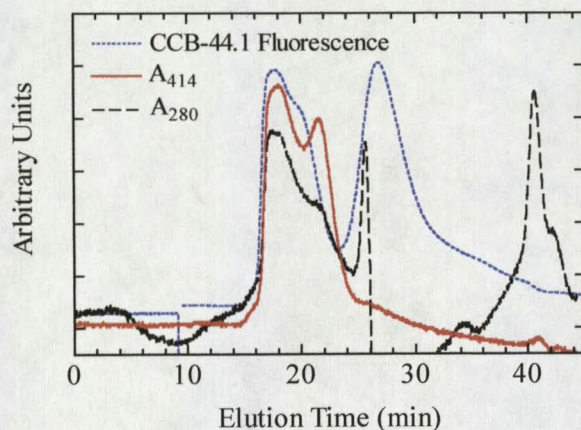


Figure 4.6. Isolation of detergent-solubilized flavocytochrome *b* complexed with CCB-44.1 by HPLC size exclusion chromatography. 100 μ L of partially-purified flavocytochrome *b* (1.4 μ M heme) was mixed with 100 μ L of 0.68 μ M (protein) CCB-

44.1, incubated for 2 h and subjected to HPLC size exclusion chromatography as described in Experimental Procedures. Shown are the elution profile chromatograms of the simultaneously measured CCB fluorescence (blue dotted line), and absorbance monitored at 280 (black dashed line) and 414 nm (red solid line). The fluorescence peak and associated A_{414} at ~ 18 min elution time corresponds to an apparent centroid mass of ~ 600 kDa relative to soluble protein standards. This mass, in combination with the absorbance and fluorescence profiles, is most consistent with a complex of 2 cytochrome *b* and 1 mAb. The fluorescence shoulder at ~ 20 min elution time corresponds to a centroid mass of ~ 420 kDa, consistent with 1 cytochrome *b* molecule complexed with one mAb. The distinct A_{414} peak eluting at ~ 22 min is free flavocytochrome *b* (~ 300 kDa), and the centroid mass of the fluorescence peak eluting at ~27 min corresponds to free mAb (~125 kDa), and has little associated A_{414} .

The mixture separated into a series of peaks with molecular masses roughly corresponding to free mAb eluting at ~ 26 min, unbound flavocytochrome *b* eluting at ~ 22 min, a 1:1 mAb:flavocytochrome *b* complex at ~ 20 min, and a 1:2 mAb:flavocytochrome *b* complex eluting at ~ 18 min. 1 min fractions were collected from the run, transferred to a cuvette and used for the following RET experiments.

Competitive Dissociation of the
CCB-44.1 Flavocytochrome *b* Complex by Mimotope Peptide.

The partial mimotope hexapeptide, PQVRPI is able to competitively inhibit mAb 44.1 from binding flavocytochrome *b* in ELISA conditions at an EC_{50} concentration of $\sim 0.5 \mu\text{M}$ (10), with nearly complete inhibition occurring at $\sim 100 \mu\text{M}$. It has also been used by our laboratory as a competitive ligand for dissociation of detergent-solubilized flavocytochrome *b* from immobilized mAb 44.1 during immunopurification (R. Taylor, *et al.*, unpublished). Addition of this hexapeptide to the HPLC fractions that contained the CCB-44.1-flavocytochrome *b* complexes should be able to dissociate the complex and consequently relax the fluorescence quenching. The HPLC fraction that eluted at ~ 18 min (Figure 4.6), corresponding to the 1:2 mAb:flavocytochrome *b* complex, was transferred to a cuvette and the fluorescence was monitored during addition of the hexapeptide PQVRPI (Figure 4.7 A). Addition of 3 equal aliquots ($5 \mu\text{L}$ each) to a final concentration of 3 mM (Figure 4.7 A, arrows) caused a reversal of the fluorescence quenching indicating competitive dissociation of the mAb-flavocytochrome *b* complex. Addition of the same concentrations of peptide to controls of either CCB-44.1 alone (Figure 4.7 B) or to the HPLC fraction that eluted at ~ 27 min, corresponding to free CCB-44.1 (Figure 4.7 A, inset), had no measurable effect on the mAb fluorescence. These results suggest that CCB-44.1 is able to form a strong complex with detergent-solubilized flavocytochrome *b*, and that the resulting quenching is due to a highly specific interaction.

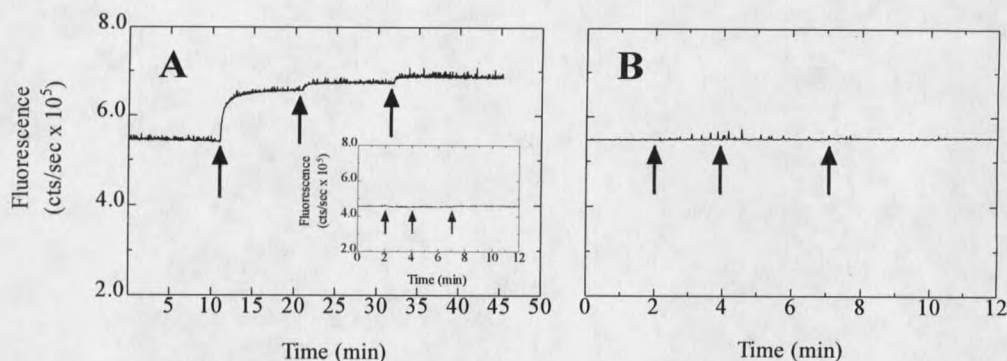


Figure 4.7. Competitive dissociation of CCB-44.1 flavocytochrome *b* complex by mimetope hexapeptide PQVRPI. **A)** Time 0 to 10 min is the fluorescence of the 17.6–18.6 min elution peak from the chromatogram shown in Figure 4.1, containing CCB-44.1 complexed with flavocytochrome *b*. Three 5 μ L aliquots of hexapeptide PQVRPI stock are added at the indicated time points (arrows), each resulting in a final concentration increase of ~ 1 mM. **Inset)** The 26.6–27.6 min elution fraction from Figure 4.1, corresponding to free CCB-44.1 is subjected to analogous conditions. Time 0 to 2 min is the initial fluorescence of the fraction. At the indicated time points (arrows) 5 μ L aliquots of hexapeptide are added to a final concentration of 3 mM, causing a very slight increase in the fluorescence intensity. **B)** A control with CCB-44.1 alone. At the indicated time points (arrows), 5 μ L aliquots of hexapeptide are added to a final concentration of 3 mM, causing a very slight increase in the fluorescence intensity.

RET Between CCB-44.1 and a Dabsylated Mimotope Peptide.

The precise locations of the CCB labeling sites on the mAbs are unknown, and the labeling stoichiometries indicate that there are dabsyl groups outside of the NH_2 -target regions. The dimensions of the IgG molecule (4,5) are such that labeling at sites distal to the antigen binding regions could easily be outside of the range where RET could occur. The fluorescence contributed by such donor molecules would not be quenched and would thus prevent accurate determination of changes in quantum yield. We reasoned that a mimotope peptide conjugated with an appropriate absorbing species could be used to determine the maximal amount of quenching that would correspond to the distance between the antigen binding site and the CCB moieties of the mAb. Such a calibration would allow for more accurate measurements to be made between the CCB moieties and the heme acceptors of flavocytochrome *b*. For this purpose we chose to covalently label the previously characterized mAb 44.1 mimotope peptide ATAGRFGGPQVNPI (10) with the chromophore 4-dimethylaminobenzene-4'-sulfonyl chloride (dabsyl). Shown in Figure 4.8 are the spectral characteristics of the chromophores for this RET system. The spectral overlap of the dabsyl group is well suited to act as an acceptor for the CCB fluorescence, characterized by a broad absorbance spectrum and a relatively high molar extinction coefficient ($24,500 \text{ M}^{-1}\text{cm}^{-1}$).

ELISA competition experiments (Figure 4.9) comparing dabsylated to non-dabsylated peptide indicated that the dabsylation had little effect on the ability of either CCB-labeled, or non-labeled mAb 44.1 to bind the conjugated peptide.

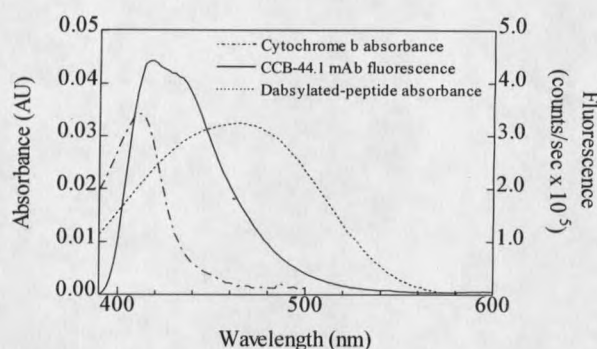


Figure 4.8. Spectral characteristics of CCB-44.1, flavocytochrome *b* heme, and dabsylated mimetope peptide ATAGRFGGPQVNPI. Shown are the superimposed normalized spectra of oxidized Soret absorbance spectrum of cytochrome *b* (—•—) ($\lambda_{\max}$ = 414 nm), the fluorescence emission spectrum of Cascade Blue[®] dye when covalently bound to mAb 44.1 (—) (λ_{ex} = 376 nm and λ_{em} maximum = 418 nm), and the absorbance spectrum of dabsyl-ATAGRFGGPQVNPI (---) ($\lambda_{\max}$ = 460 nm) in Buffer A.

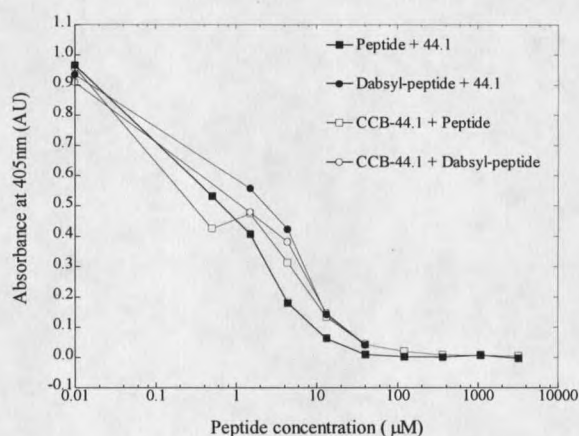


Figure 4.9. ELISA peptide inhibition of mAb 44.1 binding to immobilized flavocytochrome *b*. A dabsylated mimetope peptide ATAGRFGGPQVNPI was

compared to non-labeled peptide for its ability to competitively inhibit either CCB-labeled or non-labeled mAb 44.1 from binding immobilized flavocytochrome *b* under ELISA conditions. Traces depict competition between non-labeled mimetope peptide and non-labeled mAb 44.1 (■), dabsylated peptide and non-labeled mAb 44.1 (●), CCB-labeled mAb 44.1 and non-labeled peptide (□), and CCB-labeled mAb 44.1 and dabsylated peptide (○). IC_{50} values span ~ 1 to 3.5 μ M peptide irrespective of labeling conditions. Data shown are the results of a single experiment.

Fluorescence quenching experiments clearly demonstrated the quenching ability of the dabsylated peptide (Figure 4.10). Curve fitting analysis performed on the fluorescence quenching data indicate that at saturating concentrations of dabsyl-peptide, the fluorescence would be quenched by 49%. Förster distance calculations suggest that under such conditions the average distance between a simple donor and acceptor pair would be 50 Å. However, the antigen binding site is at most ~ 30 Å from the putative CCB-labeled NH_2 -termini of the heavy and light chains of IgG molecule. Therefore, given the labeling stoichiometry of 5.7:1 CCB:mAb, it is likely that the additional labeling sites are located at a distance greater than that separating the NH_2 -termini from the dabsyl. These results suggest that the putative CCB-labeled NH_2 -termini are quenched to a greater degree than is suggested by the 49% measured value, and that the distal labeling sites are quenched to a lesser degree, if at all.

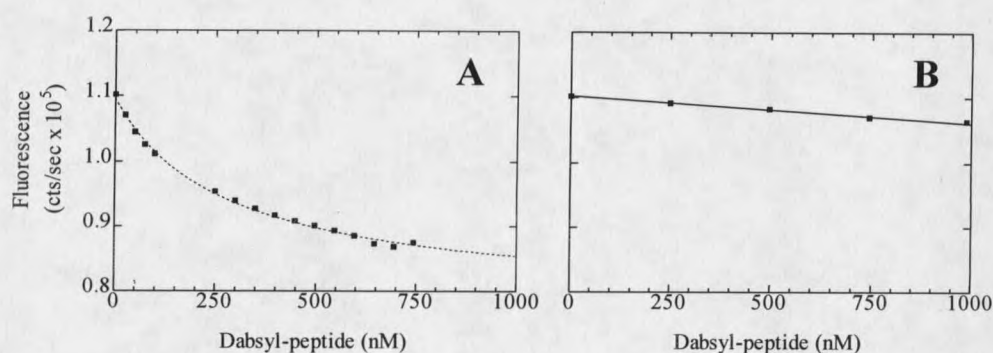


Figure 4.10. Fluorescence quenching of CCB-labeled mAb 44.1 by dabsylated mimetope peptide. **A)** Aliquots of Dabsyl-ATAGRGRRPQVNPI were added to a cuvette containing 9.46 nM (protein) CCB-44.1, the effects were allowed to equilibrate and the resulting fluorescence values were recorded (■). These data were then fit by nonlinear regression to a curve (····) modeling a 1-site binding hyperbola from which the maximum quenching was calculated to be 49% at saturating concentrations of peptide. Förster distance calculations indicate an average distance of 49.2 ± 0.2 Å between the donor acceptor pairs. The K_d for the dabsylated peptide was determined to be 280 ± 25 nM. **B)** For comparison, successive aliquots of the dabsylated peptide were added at the indicated concentrations (■) to a cuvette containing a CCB-ethanolamine conjugate with a fluorescence intensity equivalent to the CCB-44.1 shown in panel A. The resulting slight change in fluorescence illustrates the combined dilution and inner-filter effects from the addition of the dabsylated peptide up to a final concentration of 1 μ M. Data shown are from 3 separate experiments conducted on the same day.

RET Between CCB-44.1 and Flavocytochrome *b* in Isolated
Neutrophil Membranes. AA Relaxes Fluorescence
Quenching Analogous to Detergent-solubilized Flavocytochrome *b*.

Our previously discussed CCB-WGA RET system utilized detergent solubilized flavocytochrome *b*. Almost all transmembrane proteins are glycosylated, and WGA has been shown to bind several of those solubilized from neutrophil membranes (15). Application of the CCB-WGA RET system to determine the absolute levels of quenching by flavocytochrome *b* in a native lipid environment would require a high degree of purification and relipidation. The unequivocal selectivity of the mAb – epitope interaction, however, allows targeting of specific proteins in more complex environments such as neutrophil membranes, thus obviating the need for purification of the protein of interest. Although our CCB-WGA, detergent-solubilized flavocytochrome *b* RET system identified structural rearrangements in flavocytochrome *b* following exposure to oxidase agonists, the ability of such preparations to reconstitute oxidase activity *in vitro* is compromised. These effects therefore required corroboration in a system more closely resembling the cellular environment.

The CCB-labeled mAb 44.1 was thus employed to examine whether the activator-induced structural changes that we observed in our detergent-solubilized system also occurred when flavocytochrome *b* was imbedded within its native lipid environment. Figure 4.11 shows that when neutrophil membranes (final concentration ~ 15 nM heme) were added to a cuvette containing CCB-44.1 (20 nM protein), the fluorescence was partially quenched in a fashion similar to that observed in the detergent solubilized RET

system (Figure 4.11, curve 1, both panels).

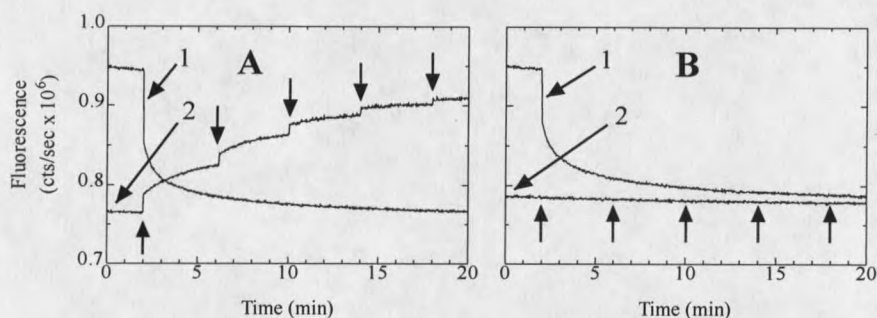


Figure 4.11. AA but not AA-ME relaxes fluorescence quenching of CCB-44.1 mAb by membrane bound flavocytochrome *b*. **A)** Time 0-2 min of curve 1 shows the initial fluorescence intensity of 20 nM (protein) CCB-labeled mAb 44.1 in cell-free oxidase Assay Buffer. At 2 min a single 10 μ L aliquot of neutrophil membranes (1×10^8 cell equivalents stock, 750 nM heme) is added to the cuvette and the fluorescence is monitored for the remainder of the 20 min run. The run is continued (curve 2) and beginning at two min, 5 successive 1 μ L aliquots of 10 mM AA are added at the indicated time points (arrows) to a final concentration of $\sim 100 \mu$ M. The fluorescence quenching is relaxed following each addition of AA. **B)** The same experiment as conducted in A, but substituting AA-ME for AA. Addition of AA-ME does not relax the quenched fluorescence.

Also in parallel with the detergent-solubilized system, addition of AA caused a concentration-dependent relaxation of the fluorescence quenching (Figure 4.11 A) with

similar saturability properties. Analogously, addition of the oxidase antagonist AA-ME failed to relax the fluorescence quenching (Figure 4.11 B) confirming the specificity of these effects. Because the membrane-bound flavocytochrome *b* used for this experiment was shown by us to be fully capable of reconstituting *in vitro* oxidase activity in the same buffer conditions, these preliminary results further support the notion of direct interactions between the activators and flavocytochrome *b* to induce functionally relevant changes its molecular geometry. The striking similarity of the interactions between these two disparate systems strongly suggests that the detergent-solubilized RET system is an appropriate model for membrane-bound, fully-functional flavocytochrome *b*. Further experiments need to be conducted to correlate the biochemical activity of the synthetic lipids with their effects on membrane-bound flavocytochrome *b*. Such a correlation would provide compelling evidence that the fluorescence quenching relaxation effects imparted by the activators represent an allosteric mechanism that is used by flavocytochrome *b* to control oxidase activity.

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CHAPTER 5

SUMMARY

The work described in this dissertation contributes new information about the structure and function of human neutrophil flavocytochrome *b*. Chapter 2 summarizes our work that provided the first direct evidence localizing the flavocytochrome *b* hemes to the NH₂-terminal, membrane-spanning regions of the protein. By combining controlled proteolysis with HPLC size exclusion chromatography we were able to isolate a fragment of flavocytochrome *b* that retained a native heme absorbance spectrum. Through a series of analyses, we determined that the fragment was composed of the NH₂-terminal 320 to 363 amino acids of gp91^{phox}, and the NH₂-terminal 169 to 171 amino acids of p22^{phox}. The structural stability of the fragment, as evidenced by its spectral characteristics, further suggested that the interfacial contact regions between the large and small subunits of flavocytochrome *b* were also preserved. The extent of glycosylation of the proteolytic fragment appeared identical to that of native gp91^{phox} suggesting that all of the sites of glycosylation were also retained.

Chapters 3 and 4 detail the exploration of the structure-function relationships of flavocytochrome *b* using the method of resonance energy transfer spectroscopy. We developed two unique systems of extrinsic fluorescence donors for this purpose by covalently labeling wheat germ agglutinin and previously characterized α -flavocytochrome *b* monoclonal antibodies with the fluorescence dye, Cascade Blue.

These systems were used to test the hypothesis of the induction of an active state geometry of flavocytochrome *b* as an integral step in control of transmembrane electron flow by the NADPH oxidase. We determined that the anionic amphiphilic oxidase activators LDS, SDS, and AA interact directly with both membrane-bound and detergent-solubilized flavocytochrome *b* causing dramatic structural changes in the protein at concentrations consistent with maximal enzyme activity. Our preliminary results in Chapter 4 additionally suggest that specific synthetic lipid analogues induce similar structural rearrangements in flavocytochrome *b*. Within the group of lipids that were tested, only those possessing an anionic head group caused effects similar to SDS, LDS, and AA. Moreover, within this group of anionic lipids, the degree of structural perturbation decreased as a function of increasing acyl chain length, thus paralleling their effectiveness to induce oxidase activation *in vitro*. Collectively, these observations suggest that 1) the structural changes in flavocytochrome *b* induced by LDS, SDS, AA, and possibly specific phosphatidic acids, are not through a random detergent-like interaction, but are dictated by their chemical and structural characteristics, and 2) these effects may reflect the induction of an active-state conformer of flavocytochrome *b*, thus establishing a link between changes in the geometry of the flavocytochrome *b* and regulation of transmembrane electron and proton conduction. The confirmation of a lipid-mediated allosteric mechanism that is unique to flavocytochrome *b* may provide new insight into the molecular control of NADPH oxidase or proton channel activity.

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