Photochemical evidence for the participation of histidine in the active center of carboxy-peptidase-Ay
by Kenneth Allen Freude

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
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Abstract:
Dye-sensitized photooxidation using either methylene blue or Rose Bengal as sensitizers rapidly diminishes both the peptidase and esterase activities of carboxypeptidase-Aγ. It is significant that both activities are simultaneously diminished by this chemical modification procedure, since heretofore a dual inactivation has been accomplished only by removal of the metal from the enzyme. All other chemical modifications employed on carboxypeptidase-A previously reported, including methylene blue sensitized photooxidation of carboxypeptidase-Aδ have resulted in an increase in esterase activity and a decrease in peptidase activity. There is a considerable difference in the mechanism of action of Rose Bengal and methylene blue. Rose Bengal functions as a very effective sensitizer for photooxidation of both carboxypeptidase-Aγ and apocarboxypeptidase-Aγ, while methylene blue sensitizes the photooxidation of only the metal free form of the enzyme. Both sensitizers show a marked pH dependence and give identical sigmoidal curves which are typical of the pH dependency of the dye-sensitized photooxidation of the imidazole moiety. Crystal violet, a sensitizer which is reported to be specific for cysteine residues, was found to be completely ineffective on apocarboxypeptidase-Aγ. Amino acid analysis of photooxidized enzyme revealed the loss of only histidine residues. Photosensitive amino acids such as cysteine, tyrosine, tryptophan, and methionine remained essentially unaltered. Significant protection against photooxidation was demonstrated using the substrates hippuryl-L-phenylalanine , glycyltyrosine, and the competitive inhibitor g-phenylpropionate. Kinetic studies indicate that Rose Bengal also functions as a competitive inhibitor of carboxypeptidase-Aγ and prevents the exchange of metal ions at the active center of the enzyme. The photolability of several metallo derivatives of carboxypeptidase-Aγ was investigated. Conformational changes subsequent to photooxidation and the photolability of the enzyme in various buffer systems were also investigated.

PHOTOCHEMICAL EVIDENCE FOR THE PARTICIPATION OF HISTIDINE IN THE ACTIVE CENTER OF CARBOXYPEPTIDASE-A_γ

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ABSTRACT

Dye-sensitized photooxidation using either methylene blue or Rose Bengal as sensitizers rapidly diminishes both the peptidase and esterase activities of carboxypeptidase-A_γ. It is significant that both activities are simultaneously diminished by this chemical modification procedure, since heretofore a dual inactivation has been accomplished only by removal of the metal from the enzyme. All other chemical modifications employed on carboxypeptidase-A previously reported, including methylene blue sensitized photooxidation of carboxypeptidase-A_δ have resulted in an increase in esterase activity and a decrease in peptidase activity. There is a considerable difference in the mechanism of action of Rose Bengal and methylene blue. Rose Bengal functions as a very effective sensitizer for photooxidation of both carboxypeptidase-A_γ and apocarboxypeptidase-A_γ, while methylene blue sensitizes the photooxidation of only the metal free form of the enzyme. Both sensitizers show a marked pH dependence and give identical sigmoidal curves which are typical of the pH dependency of the dye-sensitized photooxidation of the imidazole moiety. Crystal violet, a sensitizer which is reported to be specific for cysteine residues, was found to be completely ineffective on apocarboxypeptidase-A_γ. Amino acid analysis of photooxidized enzyme revealed the loss of only histidine residues. Photosensitive amino acids such as cysteine, tyrosine, tryptophan, and methionine remained essentially unaltered. Significant protection against photooxidation was demonstrated using the substrates hippuryl-L-phenylalanine, glycyltyrosine, and the competitive inhibitor β-phenylpropionate. Kinetic studies indicate that Rose Bengal also functions as a competitive inhibitor of carboxypeptidase-A_γ and prevents the exchange of metal ions at the active center of the enzyme. The photolability of several metallo derivatives of carboxypeptidase-A_γ was investigated. Conformational changes subsequent to photooxidation and the photolability of the enzyme in various buffer systems were also investigated.

INTRODUCTION

Carboxypeptidase-A_γ is a zinc metalloenzyme which exhibits peptidase and esterase activities.[1] The zinc atom can be reversibly removed, which results in the loss of both the peptidase and esterase activities.[2] Since the removal of the metal ion from this metalloenzyme is reversible, it should be possible to identify the metal binding site by employing various specific chemical reagents or other procedures. Such chemical modification procedures have led to the suggestion that the metal ion is bound to a thiol group of a cysteine residue and to the α-amino group of the N-terminal amino acid of carboxypeptidase-A_δ.[3]

Interpretation of the results of the methods used to detect the thiol group in this enzyme are subject to question. Reagents useful for thiol detection, such as iodo and bromoacetate, iodoacetamide and N-ethylmaleimide fail to react with the native or metal free enzyme.[3] Sulfhydryl groups were, however, determined using silver ion, p-mecuribenzoate, and ferricyanide.[3] It is interesting that the only methods which were capable of demonstrating the presence of free sulfhydryl groups in this enzyme system were methods which depended upon metal binding. This fact becomes exceedingly important when one considers that these methods were applied to a system which is known to bind metals, that is carboxypeptidase-A_γ. In addition, when carboxypeptidase was subject to reducing reagents such as sodium borohydride, or 2-mercaptoethanol, it would then form derivatives with iodoacetate and N-ethylmaleimide.[4] These results imply that the enzyme contains in its structure a disulfide linkage, instead of two chemically unreactive sulfhydryl groups.[4]

Complexometric titration[5] of apocarboxypeptidase with Zn^{2+} reveals two binding groups of apparent pK_a's of 7.7 and 9.1. The pK_a of 9.1 represents the only substantial evidence of the involvement of sulfhydryl, but by no means confirms this hypothesis. The pK_a of 7.7 would correspond to either the titratable proton of imidazole or of the N-terminal α-amino group. The involvement of imidazole seems a better choice for the reasons suggested here. Carboxypeptidase

exists in three major forms and one minor form. These forms, the alpha, beta, gamma, and delta, have identical specificities, but differ in solubility, in chain length, and in their N-terminal residues. The N-terminal residues are alanine, serine, asparagine, and asparagine, respectively. Due to the heterogeniety of the N-terminal region of the molecule, conformational differences among the various forms would be expected if the binding of zinc atoms involved participation of the N-terminal residues. These conformational differences should result in altered specificity of the various enzymatic forms, which is not observed. Further, procarboxypeptidase has been shown to bind metal.[6] Procarboxypeptidase consists of a single polypeptide chain of molecular weight 87,000, and yields one of the four carboxypeptidases-A, upon cleavage by the endopeptidases trypsin and chymotrypsin. This fact indicates that the binding site of the zinc is already established in the procarboxypeptidase, and that the N-terminus of carboxypeptidase is not involved in zinc binding. The possible involvement of imidazole in the catalytic activity of carboxypeptidase has been implied in several studies.

Since there was considerable evidence indicating that histidine could be involved in the activity of carboxypeptidase, a photochemical investigation was undertaken on the gamma form of the enzyme.

MATERIALS AND METHODS

The peptidase and esterase activities were determined in the following manner: The rate of hydrolysis of hippuryl-L-phenylalanine, (HPA), the peptide substrate, or of hippuryl-DL-phenyllactic acid, (HPLA), the esterase substrate, was measured by the increase in absorbance at 254 mμ at 25° C. A unit of activity is equal to one micromole of substrate hydrolyzed per minute under the conditions specified. The substrate solution was 0.001 M HPA or HPLA in 0.025 M Tris buffer, pH 7.5, containing 0.5 M NaCl. The change in optical density was recorded on a Gilford spectrophotometer. A full scale absorbance of 0.1 or 0.2 absorbance units was generally employed. The specific activity was determined according to the following equation:

$$\text{specific activity} = \frac{\Delta A\ 254\ m\mu / \text{min.}}{0.36 \times \text{mg enzyme/ml reaction mixture}}$$

where 0.36 equals the molar absorbance index of hippuric acid which is formed stoichiometrically.

The activity curves were obtained by pipetting a suitable aliquot of the experimental enzyme solution into 1.5 ml of substrate solution in a cuvette, mixing and placing into the spectrophotometer within 15 seconds. Carboxypeptidase-A_γ was obtained from Worthington Biochemical Corp., Freehold, New Jersey, or was prepared by the method of Anson.[27] There was no detectable difference between the commercial preparation and the enzyme prepared in this laboratory. Stock solutions were prepared by dissolving suitable quantities of the commercial enzyme in three molar sodium chloride solution after washing the crystals several times with cold deionized water. The protein concentration was determined by measuring the optical density at 278 mμ and using an absorptivity constant of 6.42×10^4 liter mole.[26]

Other chemicals were obtained from the following sources: Hippuryl-DL-phenyllactic acid (HPLA), Nutritional Biochemicals; Hippuryl-L-phenylalanine (HPA), Mann Research Laboratories; 1,10-phenanthroline (OP), J. T. Baker Chemical Co.; Cellex-T (TEAE),

Bio-Rad Laboratories; β-phenylpropionate, J. T. Baker Chemical Co.; Koshlands reagent, Cyclochemical Co.; Glycyltyrosine, Sigma Chemical; Rose Bengal and methylene blue, Fischer Scientific Co.; Crystal violet, Matheson, Coleman, and Bell; Thiopyronin, a gift from Dr. J. S. Bellin, Polytechnic Institute of Brooklyn.

The photooxidations were conducted in one of two ways. (A) A Sears 500 watt slide projector was focused at a distance of 18 to 20 cm. into the bottom of a plastic test tube which was supported at an angle in an ice bath. All manipulations of enzyme solutions containing dye were conducted in a darkened room. (B) The second method, which was found to be much more successful, was as follows: the projector was directed at a mirror held at a 45° angle to the projector lens. The projector lens was actually in contact with the mirror. Just below the mirror at a 90° angle to the projector lens was a Bell and Howell f 1.6 movie projector lens. The lens was positioned in such a manner as to allow the light to pass through in a condensing manner. This lens ensemble enabled the light to be condensed to a spot having a diameter of approximately 1.5 to 2.0 cm. The samples to be photooxidized were contained in small plastic cups which were inserted into holes in an aluminum block. The holes in the aluminum block were large enough so that the sample containing cups could be shut off from room light by means of rubber stoppers. The aluminum block was drilled to allow circulation of ice water during the determinations. The use of this block allowed consistent positioning of the sample in the light beam and permitted the use of very short exposure times. These two methods will be referred to as Method A and Method B in future discussion.

Buffer solutions were prepared with the aid of a Corning Model 12 research pH-meter. When metal ion contamination was to be avoided, all buffer solutions were extracted with a solution of dithizone in chloroform. The small amount of dissolved chloroform was removed from the buffer by bubbling purified air through the buffer at room temperature.

When plastic ware or glass ware was used to handle metalfree solutions, it was presoaked in an ethanol-EDTA bath for several hours, rinsed with ethanol, and air dried. Dialysis of solutions was conducted in special dialysis chambers. Teflon inserts were prepared to fit inside the dialysis tubing at each end. A neoprene O-ring was placed around the dialysis tubing and teflon insert to obtain a tight seal. One teflon insert was fitted with an O-ring sealed stopper which could be removed for introduction or removal of the sample. Stirring of the solution in the dialysis bag was accomplished by means of a small teflon stir bar. The entire apparatus was then suspended in the dialysis buffer, and both solutions stirred with a magnetic stirrer.

Hydrolysis of the protein mixtures were carried out in sealed Carius tubes for 20-24 hours at 110° C. Following hydrolysis an aliquot was removed and dried over KOH in vacuum. The residue was then dissolved in water and a suitable aliquot prepared and analyzed for amino acid content. Amino acid analyses were accomplished with a Technicon Amino Acid Analyzer.

PROCEDURE AND RESULTS

Photooxidation of carboxypeptidase-A_γ in the presence of 1,10-phenanthroline and methylene blue. This effect was determined by measuring the peptidase activity of the enzyme solution (0.023 mg/ml) before and after photooxidation, for both the apo and metal-containing enzyme. The apoenzyme was prepared by incubating, for up to fifteen minutes, the native enzyme at 0°, in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl and 5×10^{-4} M 1,10-phenanthroline. The measurement of peptidase activity in the apoenzyme system was accomplished by adding a slight molar excess of a 10^{-1} M $ZnCl_2$ solution to the apoenzyme system just prior to the assay. In the experimental solutions containing methylene blue the concentration of the dye was 0.002 mg/ml. Method A was used in the photooxidation. Table I summarizes the experimental composition and results of each determination.

The effect of photooxidation with methylene blue in the presence of 1,10-phenanthroline on esterase activity. Five microliters of a 3.58 mg/ml solution of carboxypeptidase-A_γ was diluted to 1.0 ml with 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl. The final concentration of carboxypeptidase was 0.023 mg/ml. The reagents 1,10-phenanthroline and methylene blue, when included in the buffer, were at concentrations of 1×10^{-3} M and 0.002 mg/ml respectively. Photooxidation was by Method A. The esterase activity was determined as described in "Methods", using hippuryl-DL-phenyllactic acid as substrate. Table II summarizes the experimental composition and results of each determination.

The effect of methylene blue-1,10-phenanthroline photooxidation of carboxypeptidase-A_γ on esterase activity. Considerable care was taken to insure that each experimental solution of carboxypeptidase was handled in an identical manner, and for the same period of time. The experimental solutions were prepared so that the final concentrations of reagents were as follows: carboxypeptidase-A_γ,

TABLE I

Photooxidation of Carboxypeptidase-Aγ in the Presence of 1,10-Phenanthroline and Methylene Blue

	μl enzyme	ml buffer	ml OP	ml MB	min hν	μl Zn^{2+}	spec. act. peptidase
A	5	1.0	—	—	—	—	56.6
B	5	0.75	0.25	—	—	—	5.5
C	5	0.75	0.25	—	—	5	50.2
D	5	0.25	0.25	0.5	—	5	44.8
G	5	0.25	0.25	0.5	30	5	24.6
H	5	0.5	—	0.5	30	—	50.2

The initial concentrations of reagents in 0.1 M Tris buffer, pH 7.0, containing 1.0 M NaCl were as follows: MB, 0.002 mg/ml; OP, 2×15^{-3} M; Zn^{2+}, 10^{-1} M. Photooxidation was by Method A, of "Methods and Materials".