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^{1}H, ^{13}C, ^{15}N backbone and side chain NMR resonance assignments of the N-terminal NEAr Iron transporter domain 1 (NEAT 1) of the hemoglobin receptor IsdB of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is an opportunistic pathogen that causes skin and severe infections in mammals. Critical to *S. aureus* growth is its ability to scavenge iron from host cells. To this effect, *S. aureus* has evolved a sophisticated pathway to acquire heme from hemoglobin (Hb) as a preferred iron source. The pathway is comprised of nine iron-regulated surface determinant (Isd) proteins involved in heme capture, transport, and degradation. A key protein of the heme acquisition pathway is the surface-anchored hemoglobin receptor protein IsdB, which is comprised of two NEAr transporter (NEAT) domains that act in concert to bind Hb and extract heme for subsequent transfer to downstream acquisition pathway proteins. Despite significant advances in the structural knowledge of other Isd proteins, the structural mechanisms and molecular basis of the IsdB-mediated heme acquisition process are not well understood. In order to provide more insights into the mode of function of IsdB, we have initiated NMR structural studies of the first NEAT domain of IsdB ($IsdB^{N1}$). Herein, we report the near complete ^{1}H, ^{13}C and ^{15}N resonance assignments of backbone and side chain atoms, and the secondary structural topology of the 148-residue IsdB NEAT 1 domain. The NMR results are consistent with the presence of eight β-strands and one α-helix characteristic of an immunoglobulin-like fold observed in other NEAT domain family proteins. This work provides a solid framework to obtain atomic-level insights toward understanding how IsdB mediates IsdB-Hb protein-protein interactions critical for heme capture and transfer.

Keywords

Staphylococcus aureus; NEAr Transporter (NEAT) domains; IsdB protein; NMR resonance assignments; protein secondary structure; bacterial heme acquisition pathway; Iron surface determinant (Isd) proteins

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Biological context

Staphylococcus aureus, a Gram-positive pathogen, is a leading cause of skin infections and life-threatening systemic infections including endocarditis and toxic shock syndrome (Casey et al. 2007). As antibiotic resistant strains of *S. aureus* emerge as a growing health threat, a better understanding of the physiology and biochemistry of this Gram-positive organism is needed. The structural knowledge gained here aims to aid in the development of new generations of therapeutics to help combat *S. aureus* infections and possibly other antibiotic resistant bacterial pathogens.

In order to initiate a successful infection, *S. aureus* requires the essential metal ion iron. In humans, the preferred source of iron originates from heme of host human hemoglobin (Skaar et al. 2004). The heme capture and acquisition by *S. aureus* is mediated by a sophisticated network of surface-accessible Iron-regulated Surface Determinant (Isd) proteins (Maresso and Schneewind 2006), which interact in a concerted way to abstract and transfer heme from hemoglobin to downstream protein effectors (Zhu et al. 2008).

The heme acquisition machinery of *S. aureus* includes the cell wall anchored surface proteins IsdA, IsdB, IsdH, IsdC and the ATP-binding cassette-like transporter IsdDEF, which are responsible for heme capture, transfer, and translocation to the bacterial cytoplasm. Once in the cytoplasm, heme is degraded by the heme monooxygenases, IsdG and IsdI and iron is assimilated into bacterial proteins (Maresso and Schneewind 2006).

The Isd proteins involved in the initial heme capture from hemoglobin and heme relay, IsdB, IsdH, IsdA and IsdC, are modular proteins and contain one or more copies of the NEAr iron Transporter (NEAT) domain (Andrade et al. 2002). IsdA and IsdC contain a single NEAT domain while the hemoglobin receptors IsdB and IsdH contain two and three, respectively (Pilpa et al. 2009). Although these domains adopt a structurally conserved 8-stranded immunoglobulin fold, they have evolved different functions outlining the functional importance of protein-protein interactions and the modular architecture of protein folds (Pilpa et al. 2006; Sharp et al. 2007; Grigg et al. 2007; Villareal et al. 2008; Watanabe et al. 2008; Gaudin et al. 2011). In the case of IsdB, the Neat 2 domain ($IsdB^{N2}$) binds heme (Gaudin et al. 2011), whereas $IsdB^{N1}$ does not. It has been speculated that $IsdB^{N1}$ binds hemoglobin to facilitate heme capture by $IsdB^{N2}$, however, detailed knowledge of $IsdB^{N1}$-hemoglobin interactions remains to be elucidated. In an effort to provide atomic-level insights into Hb capture by IsdB, determination of the high-resolution three-dimensional solution structure of $IsdB^{N1}$ has been undertaken. Herein, we report the near complete ^{1}H, ^{15}N, ^{13}C resonance assignments of backbone and side chain atoms of the N-terminal NEAT 1 domain of IsdB, $IsdB^{N1}$.

Methods and experiments

Cloning, Expression, and Purification

The gene sequence encoding the first NEAT domain of IsdB (residues Leu 125 to Asp 272 of IsdB) was amplified by PCR from a pET-21d plasmid containing the *isdb* gene encoding the mature IsdB protein as described in (Zhu et al. 2008). Forward and reverse primers used to amplify the gene sequence coding for $IsdB^{N1}$ were as follows: 5'-GACGACGACAAGATGTTGAATCAGGAACTTAGAGAAGCGAT-3' and 5'-GAGGAGAAGCCCGGTTCAATCTTCTTCAGTTTTGAATTTATCTGCA-3', respectively. The resulting PCR product was gel purified and cloned into the pET-46 Ek/LIC vector (*Novagen*). Sequencing of the cloned fragment, performed at the Iowa State University DNA Facility, confirmed the proper sequence and found no spurious mutations. The resulting $IsdB^{N1}$ protein encoded by the cloned IsdB gene fragment contained an N-

terminal 6xHis tag with the following amino acids MAHHHHHHVDDDDKM added at the N-terminus of the native $IsdB^{N1}$ sequence.

Escherichia coli BL21 (DE3) cells transformed with the $IsdB^{N1}$ encoding plasmid were grown overnight into LB medium and saved as glycerol cell stocks stored at −80°C. To produce uniformly labeled ^{15}N, and/or ^{15}N and ^{13}C-labeled $IsdB^{N1}$, a 40 mL starter culture was grown overnight in LB medium at 37°C. This culture was used to inoculate 1L of cells grown in M9 minimal media supplemented with $^{15}NH_4Cl$ (1.5g/L), or $^{15}NH_4Cl$ and ^{13}C-labeled-glucose (3.0 g/L) (i.e. D-glucose-$^{13}C_6$, 99% ^{13}C-enriched, *Cambridge Isotopes*) as the sole nitrogen and carbon sources respectively, to an initial OD_{600nm} reading of 0.1. Protein production was subsequently induced by adding 1mM IPTG to the resulting cell cultures, cells were allowed to grow for another 8 hrs prior to harvest by centrifugation at 4,000 × g (Sorvall, RC-5), and the resulting cell pellets stored at −20 °C until further use.

Cells were thawed and resuspended in 5 ml/gram of lysis buffer (20 mM Tris, 500 mM NaCl, 50 mM Na_2HPO_4/NaH_2PO_4, 10 mM imidazole, pH 8) with freshly prepared 0.1 mM PMSF. Cells were then lysed using an M-110L microfluidizer (*Microfluidics*). The resulting cell lysate was kept at 4°C and clarified from resulting cell debris by centrifugation at 12,000×g for 20 minutes. The resulting supernatant was applied to a nickel affinity chromatography column containing 5mL bed volume HisPur™ Ni-NTA Resin (*Thermo Scientific*). The column was washed with 2x the bed volume of lysis buffer and eluted with lysis buffer containing 250mM imidazole. The $IsdB^{N1}$ protein-containing fractions were pooled and dialyzed against NMR buffer (50mM Na_2HPO_4/NaH_2PO_4, 400mM sodium chloride, 1mM EDTA, 0.1mM PMSF, 0.01% sodium azide, pH 6.8), in either 95% H_2O/5% D_2O or 100% D_2O (for acquisition of 3D ^{13}C-edited ^1H-^1H TOCSY and NOESY NMR spectra), and concentrated to 1mM protein concentration as determined by OD_{280nm} readings using an centrifugal filter with 10,000 molecular weight cutoff. The purity of the protein (> 90% pure) was determined with SDS-PAGE and the molecular weight of the protein confirmed with mass spectroscopy at the Proteomics and Metabolomics Mass Spectrometry Research Facility of Montana State University. The monomeric state of $IsdB^{N1}$ was confirmed using gel filtration chromatography.

NMR Spectroscopy

All NMR spectra were acquired at 298K (25°C) on a four-channel Bruker DRX 600 spectrometer with an inverse detection triple resonance (^{15}N, ^{13}C, ^1H) conventional NMR probe equipped with triple axis gradients, as previously described for other proteins of interest (Schlenker et al. 2012; Tripet et al. 2011). All data were processed and analyzed using NMRPipe Spectral Processing and Analysis System (Delaglio et al. 1995) and Sparky NMR Assignment and Integration Software (Goddard and Kneller 2008). Sequential $^{15}N/^1H/^{13}C$ backbone and side chain resonance assignments were extracted from standard heteronuclear (^1H, ^{15}N, ^{13}C) multidimensional NMR experiments (HNCA, HNCACB, CBCA(CO)NH, C(CO)NH, HBHA(CO)NH, HC(CO)NH, HN-TOCSY, ^{13}C-edited ^1H-^1H TOCSY, ^{13}C-^1H HSQC, and ^{13}C-edited and ^{15}N-edited ^1H-^1H NOESY). ^1H, ^{15}N, and ^{13}C chemical shift dimensions were indirectly referenced to DSS.

Extent of NMR assignments and data deposition

NMR data collected on the recombinant $IsdB^{N1}$ protein enabled resonance assignments of 93% all assignable $^1H^N$ and ^{15}N resonances (132 out 142, and excluding all 7 prolines of the protein sequence) (Fig. 1). Ten backbone amide resonances could not be assigned due to significant resonance broadening or extensive spectral overlap. Four of the missing assignments correspond to aromatic residues $F_{164}Y_{165}H_{166}Y_{167}$. Based on sequence homology, these residues are in equivalent positions to a stretch of contiguous aromatic

residues ($Y_{125}Y_{126}H_{127}F_{128}F_{129}$) of $IsdH^{N1}$ (the closest homolog to $IsdB^{N1}$) that are shown to be located in a disordered region and also unassigned in the NMR solution structure of $IsdH^{N1}$ (Pilpa et al. 2006). Interestingly, these aromatics residues in $IsdH^{N1}$ are part of the hemoglobin-binding surface (Pilpa et al. 2006; Kumar et al. 2011), suggesting that equivalent residues of $IsdB^{N1}$ could also be participating in Hb binding to the IsdB receptor. The completeness of the remaining backbone resonance assignments corresponded to: 97% of $^{13}C_{\alpha}$ (144/149), 95% of $^{1}H_{\alpha}$ (142/149), and 97% of $^{13}C_{\beta}$ (139/144). The side chains were assigned to greater than 87% completeness. Several aromatic side-chain proton resonances were assigned, but not all.

Chemical shift index (CSI) analysis (Wishart and Sykes 1994) of $IsdB^{N1}$ NMR data together with backbone ϕ and ψ dihedral angles predicted by TALOS+ from chemical shift data (Shen et al. 2009) delineated the presence of 8 β-strands spanning residues 150–156 (β1); 175–178 (β2); 183–189 (β3); 198–202 (β4); 208–215 (β5); 221–227 (β6); 223–242 (β7); 252–256 (β8), and a single α-helical segment spanning residues 128–134 (Fig. 2). The 8 β-strands point to a similar overall fold of other published NEAT domains, including $IsdH^{N1}$. Assignments of sequential and long range ^{1}H-^{1}H NOEs are now in progress and ultimately will be used to establish the high-resolution 3D structure of $IsdB^{N1}$ in solution.

Backbone and side-chain (^{1}H, ^{15}N, ^{13}C) NMR resonance assignments for $IsdB^{N1}$ have been deposited in the BioMagResBank (http://www.bmrb.wise.edu) under the BMRB accession number 19056.

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Abbreviations

PMSF	phenylmethylsulfonyl fluoride
EDTA	ethylenediamenetetraacetic acid
DSS	4,4-dimethyl-4-silopentane-1-sulfonic acid
IPTG	isopropyl-thio-β-galactoside
IsdB	Iron surface determinant protein B
$IsdB^{N1}$	N-terminal NEAr iron transporter domain 1 (NEAT 1) of IsdB
$IsdH^{N1}$	NEAT 1 domain of IsdH

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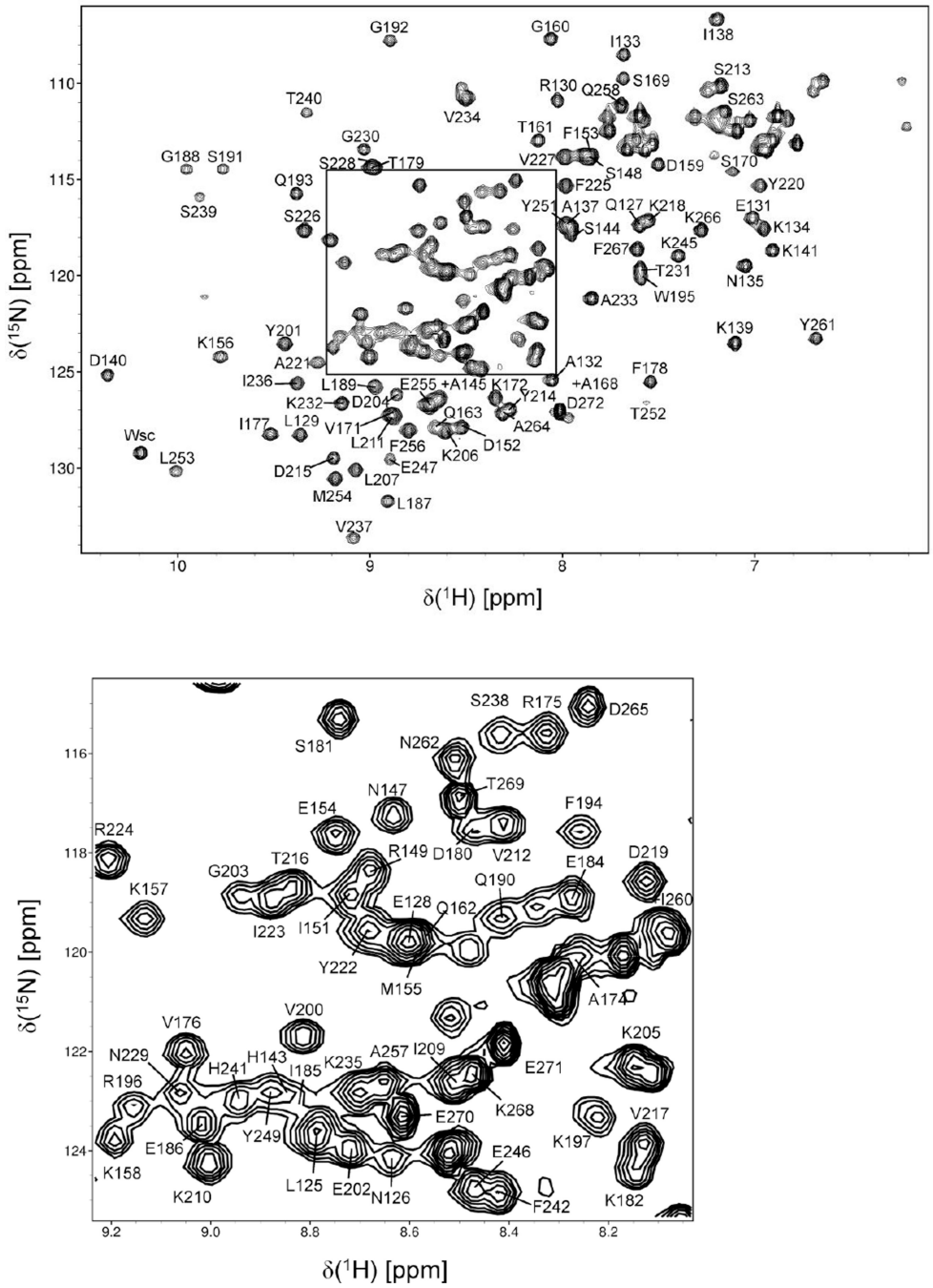

Fig. 1.

(a) 2D ^{1}H-^{15}N HSQC spectrum of 1mM IsdB^{N1} in 50mM $NaHPO_4/NaH_2PO_4$, pH 6.8, 400mM NaCl, 1mM EDTA, 0.1mM PMSF, 0.01% sodium azide, in 95% H_2O and 5% D_2O. The spectrum was recorded at 298K on a Bruker DRX 600 MHz spectrometer. Backbone resonance assignments are indicated with the *one-letter* amino acid code and residue number. The tryptophan indole NH side chain signal is marked with "Wsc." A "+" in front of residue numbers marks ^{1}H/^{15}N resonances that are too weak to be observed at the current intensity level of the displayed spectrum. Resonances from side chain NH_2 are not labeled.

(b) Insert: a close-up view of the congested middle region of the HSQC spectrum with assigned NH resonances labeled.

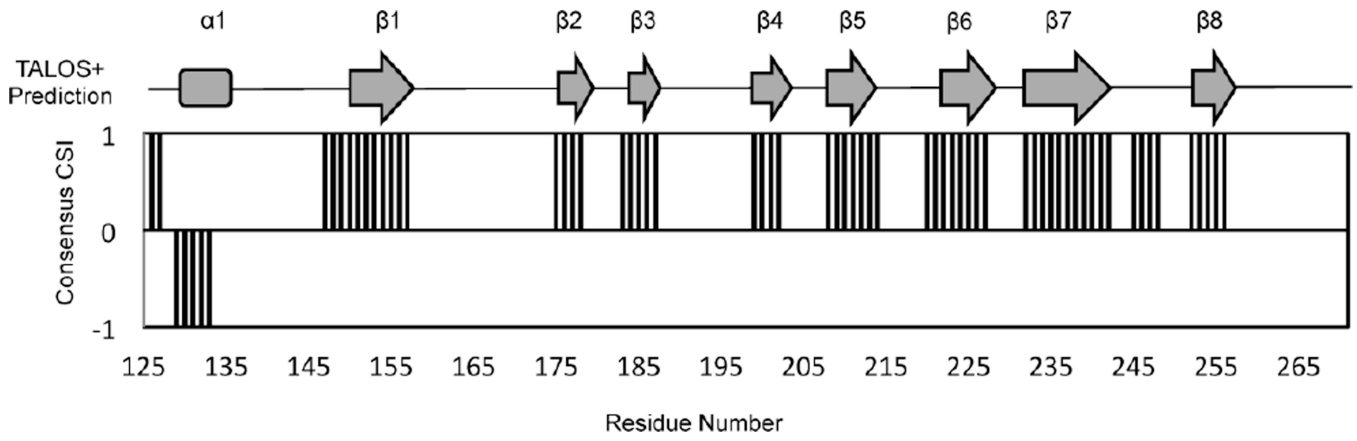

Fig. 2.

Secondary structural topology of $IsdB^{N1}$ derived from chemical shift indexing and dihedral angles predictions from TALOS+ analysis of chemical shift data. The α-helix and 8 β-strands of $IsdB^{N1}$ are shown above the CSI and TALOS+ predictions as a rectangle and arrows, respectively. The consensus chemical shift index (CSI) plot shown below $IsdB^{N1}$ secondary structural elements is based on the CSI analysis of all Hα, Cα, and Cβ chemical shifts.