Selective binding of antimicrobial porphyrins to the heme-receptor LsdH-NEAT3 of *Staphylococcus aureus*

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Abstract: The Lsd (iron-regulated surface determinant) system of the human pathogen *Staphylococcus aureus* is responsible for the acquisition of heme from the host organism. We recently reported that the extracellular heme receptor LsdH-NEAT3 captures and transfers noniron antimicrobial porphyrins containing metals in oxidation state (III). However, it is unclear if geometric factors such as the size of the metal (ionic radius) affect binding and transfer of metalloporphyrins. We carried out an ample structural, functional, and thermodynamic analysis of the binding properties of antimicrobial indium(III)-porphyrin, which bears a much larger metal ion than the iron(III) of the natural ligand heme. The results demonstrate that the NEAT3 receptor recognizes the In(III)-containing PPIX in a manner very similar to that of heme. Site-directed mutagenesis identifies Tyr642 as the central element in the recognition mechanism as suggested from the crystal structures. Importantly, the NEAT3 receptor possesses the remarkable ability to capture dimers of metalloporphyrin. Molecular dynamics simulations reveal that LsdH-NEAT3 does not require conformational changes, or large rearrangements of the residues within its binding site, to accommodate the much larger (heme)₂ ligand. We discuss the implications of these findings for the design of potent inhibitors against this family of key receptors of *S. aureus*.

Keywords: metal recognition; metalloporphyrin; antimicrobial compound; Lsd system; isothermal titration calorimetry; molecular dynamics simulations; protein–heme interaction

Abbreviations: Lsd, iron-regulated surface determinant; ITC, isothermal titration calorimetry; NEAT, near transporter; PPIX, protoporphyrin IX.

Additional supporting information may be found in the online version of this article.

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Introduction

Bacterial pathogens, such as *Staphylococcus aureus*, face severe restrictions of the essential nutrient iron during the progress of infection.^{1–4} To overcome this limitation, *S. aureus* have evolved sophisticated mechanisms to steal iron from the host organism. Notably, the iron-regulated surface determinant (Isd) system is activated in response to iron depletion.⁵ Isd comprises a group of proteins that capture, transport, and metabolize heme molecules to extract the iron atom contained in it.^{5–8} Heme is taken from hemoglobin by extracellular receptors IsdH or IsdB, and subsequently transferred to transporters IsdA and IsdC (Fig. 1). The transmembrane complex IsdEF-FhuD translocates the heme molecule (received from IsdC) across the plasma membrane, after which the cytoplasmic heme-oxygenases IsdG and IsdI degrade heme molecules.

The extracellular transporters display one or more copies of the conserved *near transporter* (NEAT) domain. For example, the first receptor IsdH destabilizes human hemoglobin using its NEAT1 and NEAT2 domains, facilitating the subsequent capture of heme by the NEAT3 domain.⁹ X-ray crystallography has showed that the heme-binding NEAT domains of IsdH, IsdA, IsdB, and IsdC are very well conserved, exhibiting a characteristic hydrophobic pocket suitable to bind heme. A conserved Tyr residue in the proximal axial position coordinates the iron atom.^{10–12} The mechanism of heme transfer between Isd transporters involves transient protein–protein interactions between the NEAT transporters.^{13–16} NEAT domains are not unique to *S. aureus*, but also appear in other pathogenic bacteria such as *Bacillus anthracis*.^{17,18}

NEAT transporters also bind noniron metalloporphyrins—that is, porphyrins bearing a metal other than iron.^{19–22} The crystal structures of the complex between metalloporphyrins and IsdH-NEAT3²¹ and between Co(III)-protoporphyrin IX (PPIX) and IsdA reveal a binding mode very similar to that of the natural ligand heme.^{20,21} Importantly, noniron porphyrins are effective antimicrobial agents against Gram-positive and Gram-negative bacteria including the human pathogen *S. aureus*.^{23,24} It has been proposed that these compounds reach the interior of the cell by a Trojan-horse mechanism using heme conduits.²³ Isd system, being the major portal of heme in *S. aureus*, is a reasonable entry pathway for antimicrobial porphyrins, although this hypothesis has not been verified in a more physiological environment.

Noniron porphyrins are also attractive molecules from pharmacological and biotechnological points of view. Noniron porphyrins have been extensively used as inhibitors of the enzyme heme oxygenase 1.^{25,26} Also, a metalloporphyrin-substituted cytochrome P450 was used as a contrast agent for

Figure 1. Isd system of *Staphylococcus aureus*. Heme molecules are sequestered from the host organism by cell-surface receptors IsdH and IsdB, and subsequently transfer to intermediate transporters IsdA and IsdC across the cell wall. Before reaching the plasma membrane transporter IsdF, the molecule of heme is transferred from IsdC to IsdE. Upon translocation across the membrane, cytosolic enzymes IsdG and IsdI extract the iron atom contained in the molecule of heme. The function of IsdD is unknown. Hm, MetHb, Hb, and Hp refer to heme, methemoglobin, hemoglobin, and haptoglobin, respectively.

magnetic resonance imaging.²⁷ Therefore, understanding the principles of the interaction between proteins and metalloporphyrins will benefit the biomedical and biotechnological research communities.

In a recent study, we showed that antimicrobial porphyrins containing a metal in oxidation state (III) (Ga^{3+} , Mn^{3+}), but not in oxidation state (II) (Zn^{2+} , Mg^{2+} , Cu^{2+}), closely mimicked the binding properties of the natural ligand heme to the receptor IsdH-NEAT3.²¹ However, it was not clarified if the specific binding properties of metal(III)-PPIX are owing to (i) the similar size of the metal ions (less than 5% difference among Fe^{3+} , Ga^{3+} , and Mn^{3+}),²⁸ or (ii) preferential interaction between metal(III)-PPIX and the tyrosinate ligand of IsdH-NEAT3.

Herein we have employed In(III)-PPIX, displaying a large metal ~25% larger than iron²⁸, to distinguish between these two mechanisms. We report that the interaction parameters of heme and In(III)-PPIX with the NEAT3 receptor are virtually identical to each other from multiple points of view (binding, structure, and transport). We verify this conclusion by using isothermal titration calorimetry. Moreover, mutation of the axial tyrosine abrogates the binding of metalloporphyrins to the receptor. We discuss the relevance of these results for the design

Figure 2. Binding of In(III)-PPIX to IsdH-NEAT3. UV-visible spectra of In(III)-PPIX (10 μ M) in the presence of increasing concentrations of receptor IsdH-NEAT3. The concentrations of NEAT3 receptor are given. Representative curves with heme are shown in the inset. The absorption peak centered $\sim$ 280 nm corresponds to protein.

and optimization of more potent antimicrobial metalloporphyrins.

Results

Binding and transfer of In(III)-PPIX

To gain insight into the mechanism of porphyrin binding to IsdH-NEAT3, we have employed In(III)-PPIX, bearing a metal $\sim$ 25% larger than the iron(III) atom of the natural ligand heme.²⁸ The binding of In(III)-PPIX to IsdH-NEAT3 produces notable changes in the Soret region of the UV-visible spectrum (Fig. 2). The molar absorptivity increases to a saturation level in the presence of increasing concentrations of receptor (up to 15 μ M). Simultaneously, the position of λ_{max} shifts from 409 nm to 417 nm. In addition, the Q bands are displaced to lower wavelengths upon progressive binding to the receptor (552 $\rightarrow$ 548 nm and 591 $\rightarrow$ 588 nm, supporting information Fig. S1). Similarly, the intensity of the Soret band of heme increased, and the position of

λ_{max} shifted from 390 nm to 401 nm (Fig. 2, inset) in agreement with previous reports.^{21,22}

Next, the transfer of In(III)-PPIX and heme from IsdH-NEAT3 to the downstream receptor apo-IsdA was examined by a standard methodology (Fig. 3).^{13,21} The intensity of the Soret band of the metalloporphyrin bound to holo-IsdH-NEAT3 decreased progressively as the incubation period with apo-IsdA increased. These results indicate productive transfer of the porphyrin moiety as previously reported for heme, Ga(III)-PPIX, and Mn(III)-PPIX.²¹ The relative amount of In(III)-PPIX attached to IsdH-NEAT3 decreased to relative values of 43% and 2% after incubation with apo-IsdA for 2 and 5 min, respectively. Similarly, the relative peaks of heme bound to NEAT3 receptor was reduced to 52% and 12% upon incubation with apo-IsdA.

Crystal structure of the complex In(III)-PPIX-IsdH-NEAT3

The crystal structure of In(III)-PPIX bound to IsdH-NEAT3 was determined by X-ray crystallography to a resolution of 2.8 Å (Table I). To the best of our knowledge, this is the first reported structure of In(III)-PPIX in complex with a protein. The sigma-A 2Fo-Fc electron density map and the omit difference density map both indicate that only one molecule of porphyrin is bound to the receptor (Fig. 4 and supporting information Fig. S2). The metal In(III) forms a coordination bond with the proximal ligand Tyr642 of the protein at a distance of 2.1 ± 0.4 Å. The distal coordination site is vacant.

The crystal structure of the complex of IsdH-NEAT3 with In(III)-PPIX bound is virtually identical to that of the receptor with heme (Fig. 5).¹² The difference between their relative atomic coordinates is very small (rmsd = 0.36 ± 0.2 Å). The residues belonging to the binding site (within 4 Å of the porphyrin moiety) also exhibit very similar conformations. The distance between the metal and Tyr642 in each structure are indistinguishable within experimental error (distance

Figure 3. Transfer of metalloporphyrin from IsdH-NEAT3 to IsdA. (A) Schematic representation of experiment. Holo-IsdH-NEAT3 and apo-IsdA are incubated together and subsequently separated by ion-exchange chromatography. (B) Experiment with heme and (C) In(III)-PPIX. UV-visible spectra of IsdH-NEAT3 after the separation step. Traces correspond to IsdH-NEAT3 in the absence of IsdA (solid line), after 2-min incubation (dotted line), and after 5-min incubation (dashed line).

Table I. Data Collection and Refinement Statistics

In(III)-PPIX	
Data collection	
Space group	P 2 ₁ 2 ₁ 2
Unit cell	
Dimensions (Å)	$a = 75.80; b = 71.01; c = 49.46$
Angles (°)	$\alpha = \beta = \gamma = 90$
Resolution (Å)	41.4 – 2.80
Wavelength	1.000
Observations	32,907
Unique reflections	6,932
$R_{p.i.m.}$ (%) ^a	0.073 (0.21)
I/σ (I)	8.1 (3.8)
Multiplicity	4.7 (4.8)
Completeness (%)	99.5 (99.8)
Refinement statistics	
Resolution (Å)	41.4 – 2.80
Reflections work/free	6,589/328
R_{work}/R_{free} (%) ^b	22.7/29.2
No. of atoms	1,951
No. of protein chains	2
No. of residues	226
No. of In(III)-PPIX	2
No. of ions/ligands	2
No. of waters	18
Protein B-factor (Å ²)	30.2
PPIX ligand B-factor (Å ²)	20.8
Ions/ligands B-factor (Å ²)	30.9
Water B-factor (Å ²)	23.5
Ramachandran Plot	
Preferred regions (%)	83.3
Allowed regions (%)	16.7
Outliers (%)	0
RMSD bond (Å)	0.012
RMSD angle (°)	1.8
PDB identification code	3VTM

Statistical values given in parenthesis refer to the highest resolution bin.

^a $R_{p.i.m.} = \sum_{hkl} (I(n_{hkl} - 1))^{1/2} \sum_i |I(hkl)_i - [I(hkl)]| / \sum_{hkl} \sum_i I(hkl)$.

^b $R_{work} = \sum_{hkl} |F(hkl)_o - [F(hkl)_c]| / \sum_{hkl} F(hkl)_o$; R_{free} was calculated as R_{work} , where $F(hkl)_o$ values were taken from 5% of data not included in the refinement.

of $\text{Fe}^{3+} - \text{Tyr642} = 2.2 \pm 0.15 \text{ Å}$; distance $\text{In}^{3+} - \text{Tyr642} = 2.1 \pm 0.4 \text{ Å}$.

Thermodynamic analysis by ITC

Deconvolution of binding phenomena into its basic thermodynamic elements is an incisive methodology to analyze ligand–protein interactions.³⁰ ITC was used to characterize the binding parameters of metalloporphyrins to NEAT3 receptor (Fig. 6 and Table II). The titration of heme with IsdH-NEAT3 is exothermic and shows a single transition with high affinity ($K_D = 39 \pm 4 \text{ nM}$). The affinity determined with the NEAT3 domain alone is not expected to change substantially with respect to longer constructs containing the NEAT2 and linker domains, as recently reported.⁹ Binding is driven by a favorable change of enthalpy ($\Delta H^\circ = -18.2 \text{ kcal mol}^{-1}$) and opposed by an unfavorable change of entropy ($-T\Delta S^\circ = 8.1 \text{ kcal mol}^{-1}$). The value of the stoichiometry parameter indicates that two molecules of heme bind to the NEAT3 receptor ($n = 2.0 \pm 0.1$). The same stoichiometry value was observed in the reverse titration of heme with protein ($n = 2.05 \pm 0.08$, not shown). The steep transition of the binding isotherm rules out unspecific binding. Because IsdH-NEAT3 has one binding site, the ITC data indicates that the ligand must bind as a dimer (heme)₂, which is the predominant species in the high-concentrated solutions employed for the ITC experiments.^{31,32} This is the first evidence of binding of (heme)₂ to a NEAT domain, although no crystal structure of such complex has been reported yet. Furthermore, the binding of (heme)₂ is also observed in a second NEAT domain, IsdA-NEAT ($n = 2.1 \pm 0.1$, supporting information Fig. S3). In contrast, when the incubation time is increased from minutes (as in the titration experiments above) to several hours,

Figure 4. Crystal structure of IsdH-NEAT3 with In(III)-PPIX bound. (A, B) Sigma-A weighted 2Fo – Fc electron density map contoured at 1.0 σ (blue mesh) or 10 σ (magenta mesh). The metal is depicted in dark purple. The carbon atoms of the porphyrin ring and the protein are depicted in yellow and orange, respectively. Oxygen and nitrogen are shown in red and dark blue. The average distance between In(III) and the coordinating Tyr642 is also indicated. Coordinate error was calculated by the method of Cruickshank.²⁹

Figure 5. Comparison of the crystal structures of IsdH-NEAT3 with heme or with In(III)-PPIX. (A) Superposition of the overall structures ($\text{rmsd} = 0.36 \pm 0.2 \text{ \AA}$). Blue and orange ribbons correspond to complexes with heme and In(III)-PPIX, respectively. The crystal structure of IsdH-NEAT3 with heme bound was retrieved from the PDB (accession code 2Z6F). In(III)-PPIX is depicted in yellow. (B) Comparison of binding sites. Heme and In(III)-PPIX are depicted in blue and orange, respectively. Iron (red) and indium (purple) are depicted as spheres. Residues within 4.0 Å of the porphyrin molecule are shown.

followed by removal of excess heme, the stoichiometry approaches unity (supporting information Fig. S3). This observation suggests that the second heme molecule of the dimer (heme_2) is loosely attached to IsdH-NEAT3 (see molecular MD simulations below) and slowly dissociates from the complex.

The binding of noniron porphyrins In(III)-PPIX, Ga(III)-PPIX, and Mn(III)-PPIX, all displaying metals in oxidation state (III), was also examined by ITC (Fig. 6, Fig. 7, and Table II). The affinities

values of In(III)-PPIX and Ga(III)-PPIX are about twofold lower than that of heme, which translates in small losses of free energy ($\Delta\Delta G^\circ_{\text{In(III)}} = 0.3 \pm 0.4 \text{ kcal mol}^{-1}$; $\Delta\Delta G^\circ_{\text{Ga(III)}} = 0.4 \pm 0.5 \text{ kcal mol}^{-1}$). The affinity of Mn(III)-PPIX, although strong ($480 \pm 46 \text{ nM}$), is noticeably lower than that of heme (12-fold lower, $\Delta\Delta G^\circ_{\text{Mn(III)}} = 1.5 \pm 0.4 \text{ kcal mol}^{-1}$). The value of the stoichiometry parameter for each of the three metalloporphyrins varied between 1.9 and 2.5 (Table II), which is consistent with the attachment

Figure 6. Binding isotherm obtained by ITC. (A) Binding isotherm of heme, (B) In(III)-PPIX, and (C) Zn(II)-PPIX. IsdH-NEAT3 (250 μM) was injected stepwise into a cell containing fresh metalloporphyrin (50 μM) at a constant temperature of 25°C. Upper panel corresponds to the time-course of the titration (black) and baseline (gray). The bottom panels show the integrated isotherms. Thermodynamic parameters were obtained by non-linear regression to a one-site binding model with the program ORIGIN.

Table II. Thermodynamic Parameters of Binding of Metalloporphyrins to IsdH-NEAT3^a

Protein	Metal	K_D (nM)	ΔG° (kcal mol ⁻¹)	ΔH° (kcal mol ⁻¹)	$-T\Delta S^\circ$ (kcal mol ⁻¹)	n^b
WT	Fe(III)	39 ± 4.0	-10.1 ± 0.2	-18.2 ± 0.1	8.1	2.0 ± 0.1
WT	In(III)	66 ± 11	-9.8 ± 0.2	-19.8 ± 0.2	10.0	2.5 ± 0.1
WT	Ga(III)	83 ± 21	-9.7 ± 0.3	-14.9 ± 0.2	5.2	1.9 ± 0.1
WT	Mn(III)	480 ± 46	-8.6 ± 0.2	-11.6 ± 0.2	3.0	2.1 ± 0.1
WT	Zn(II)	ND ^c	ND	ND	ND	ND
WT	Cu(II)	ND	ND	ND	ND	ND
WT	Mg(II)	ND	ND	ND	ND	ND
Y642A	Fe(III)	>45,000	<-5.9	ND	ND	>15
Y642A	In(III)	>300,000	<-4.8	ND	ND	>30

^a Temperature, 298 K.^b n refers to the molar ratio porphyrin/protein.^c ND, binding not detected.

of porphyrin dimers. The dissection of the energetic parameters indicates that the small loss of affinity of In(III)-PPIX with respect to heme is caused by a loss of entropy ($-T\Delta\Delta S^\circ_{\text{In(III)}} = 1.9$ kcal mol⁻¹), although this effect is minimized by a gain of enthalpy ($\Delta\Delta H^\circ_{\text{In(III)}} = -1.7 \pm 0.3$ kcal mol⁻¹).^{33,34} The lower affinity of Ga(III)-PPIX and Mn(III)-PPIX arises from less favorable interactions with the receptor ($\Delta\Delta H^\circ_{\text{Ga(III)}} = 3.3 \pm 0.3$ kcal mol⁻¹; $\Delta\Delta H^\circ_{\text{Mn(III)}} = 6.6 \pm 0.3$ kcal mol⁻¹).

In contrast to metal(III)-PPIX, we did not detect robust binding of porphyrins carrying a metal in oxidation state (II), that is, Zn(II)-PPIX, Mg(II)-PPIX, and Cu(II)-PPIX (Table II). For example, the titration of Zn(II)-PPIX with the receptor yields very little exothermic heat, rendering a binding curve without a clear transition and thus unsuitable for fitting to a wiseman isotherm.³⁵ The same features are observed with the other two porphyrins carrying metals in oxidation state (II) (not shown). These results are consistent with poor binding as previously observed by size-exclusion chromatography and UV-visible spectroscopy.²¹

The significance of the coordination bond between the metal ion and the side chain of Tyr642 was evaluated by site-directed mutagenesis. Mutein Y642A was expressed and purified to homogeneity. Titration of either heme or In(III)-PPIX with this mutein results in meager binding responses (supporting information Fig. S4). The fitting of the wiseman isotherm with the program ORIGIN indicates unspecific binding (stoichiometry > 15) and low affinity (high μM range, Table II).

Model of porphyrin dimers in complex with IsdH-NEAT3

A model of (heme)₂ bound to IsdH-NEAT3 was obtained by a combination of high-level computational calculation at the B3LYP/6-31G(d) level and molecular dynamic simulations (Fig. 8). The first molecule of the dimer, heme1, occupies a position in the binding pocket that is very similar to that of

heme in the crystal structure. Heme1 establishes multiple interactions with the receptor, including the crucial coordination bond between Tyr642 and the iron atom. The contact surface area between heme1 and IsdH-NEAT3 changes little in comparison with that in the crystal structures (Table III). The average contact interface between metalloporphyrin and IsdH-NEAT3 calculated from four independent crystal structures is 440 ± 7 Å², whereas that calculated from the model between heme1 and receptor is 396 Å². The second molecule of heme, on the contrary, displays a small interaction surface area with the receptor (162 Å²).

Importantly, the protein does not require structural reconfigurations to capture the dimer (heme)₂ [Fig. 8(B)]. The difference between the relative atomic coordinates of the protein in complex with heme (PDB entry code 2Z6F) and that in complex with (heme)₂ is very small (rmsd = 0.52 Å). Indeed, the rmsd value is only slightly larger than that calculated between the structures with heme and with In(III)-PPIX (rmsd = 0.36 ± 0.2 Å; Fig. 5).

Figure 7. Dissection of the thermodynamic parameters. Comparison of free energy (ΔG°), enthalpy (ΔH°), and entropy ($-T\Delta S^\circ$) of binding of metalloporphyrins bearing a metal in oxidation state (III). Black, gray, and white bars corresponds to ΔG° , ΔH° , and $-T\Delta S^\circ$, respectively. The identity of the metal in the porphyrin ring is given in the panel.

Figure 8. Model of (heme)₂ bound to IsdH-NEAT3. (A) The molecular model of (heme)₂ bound to IsdH-NEAT3 was obtained by molecular dynamic simulations. One molecule of heme coordinates to the receptor through residue Tyr642. The (heme)₂ moiety is stabilized by metal-propionate coordination bonds, and $\pi\cdots\pi$ and van der Waals interactions. (B) The binding of the (heme)₂ molecule to IsdH-NEAT3 does not require significant conformational changes on the protein. The model of the complex of IsdH-NEAT3 with (heme)₂, and the X-ray structure with heme bound (PDB entry code 2Z6F) were superimposed with COOT (rmsd = 0.52 Å).

Discussion

Basis of binding selectivity of metalloporphyrins

Noniron metalloporphyrins exhibit antimicrobial properties against a broad range of pathogens.^{23,24} We have recently reported that metalloporphyrins bearing Ga(III) or Mn(III), but not a metal in oxidation state (II), closely mimic the binding properties of the natural ligand heme of IsdH-NEAT3.²¹ In that study we could not determine if the mechanism explaining the selectivity of IsdH-NEAT3 was related to the similar ionic radii among the metal(III) ions of the porphyrin or to the preferential coordination to the axial ligand of the metal(III) over the metal (II) of the porphyrin. For example, the ionic radii of Ga(III) and Fe(III) only differ by 0.02 Å (0.62 Å and 0.64 Å, respectively).^{23,28,36,37}

We distinguished between these two alternatives by using indium(III), which is an ion ~25% larger than iron(III) and gallium(III).²⁸ Indium displays a stable oxidation state (III) when

incorporated in the porphyrinate molecule.³⁸ Binding assays (UV-visible and ITC), X-ray crystallography, and functional assays indicates that In(III)-PPIX closely mimic the properties of heme. In agreement with our previous report,²¹ no stable binding of metalloporphyrins bearing a metal in oxidation state (II) was detected by ITC. The selectivity is achieved through preferential coordination with the axial ligand Tyr642, since the mutation of this residue abolishes the specific interactions with the ligand (supporting information Fig. S4). This tyrosine is proposed to coordinate the porphyrin ligand as a tyrosinate ion.³⁹ The presence of a positive charge in the porphyrin ring, such as that occurring in porphyrins bearing metals in oxidation state (III), will enhance the electrostatic interactions between the tyrosinate moiety and the metal. The electrostatic interaction do not occur with metals in oxidation state (II). This mechanism seems to operate also in other extracellular Isd proteins because mutations of the axial tyrosine of IsdA and IsdB also disrupts heme binding.^{20,40}

Table III. Contact Surface Area Between IsdH-NEAT3 and Metalloporphyrin

PPIX	Surface area of PPIX-protein (Å ²)	Buried surface with respect to total surface of PPIX (%)
Heme	447	66
In(III)	433	64
Ga(III)	435	63
Mn(III)	445	65
Average	440	64
Heme1 of dimer	396	54
Heme2 of dimer	162	19

Binding of (heme)₂ to IsdH-NEAT3

We constructed a model of (heme)₂ in complex with IsdH-NEAT3 (Fig. 8) to understand the structural basis of the stoichiometry 2:1 observed in the ITC experiment (Fig. 6 and Table II). We argue that the binding of the (heme)₂ molecule is kinetically favored under the high-concentration conditions necessary for the ITC experiment because of its long dissociation constant in the scale of tens of minutes.³¹ When the incubation time is extended to

hours (or weeks, as in the crystallization experiment) the heme dimer dissociates, leading to equimolar stoichiometries in solution (supporting information Fig. 3SA) and the crystal structure (Fig. 4).^{12,21}

Except for the second molecule of heme, the simulated model of (heme)₂ bound to NEAT3 do not greatly differ from the X-ray crystal structures of IsdH-NEAT3 with heme,¹² Ga(III)-PPIX, Mn(III)-PPIX,²¹ and In(III)-PPIX (Fig. 4). Although the relevance of this binding mode has not been demonstrated in the environment of the cell, it reveals a significant flexibility of the binding pocket of the receptor with important implications for the design of inhibitors (see below). The NEAT domain of transporter IsdA also binds (heme)₂ (supporting information Fig. S3B). These observations add NEAT domains to a growing number of bacterial binding proteins of bacterial origin capable of binding two molecules of heme *in vitro*, such as Shp from *Streptococcus pyogenes*,⁴¹ HmuT from *Yersinia pestis*,⁴² and MhuD from *Mycobacterium tuberculosis*.⁴³ Moreover, the striking analogies between the (heme)₂ molecule and the primary building block of microcrystalline β -hematin, also known as malaria pigment or hemozoin,^{44,45} suggest that NEAT3 receptor may recognize this key compound accumulated during the infective cycle of *Plasmodium falciparum*.

Implications for the design of stronger antimicrobial porphyrins

Noniron metalloporphyrins are a family of antibacterial compounds of therapeutic potential. It has been suggested that these compounds enter the cell by a Trojan-horse mechanism using the native heme-transporters of the bacterium.^{23,46} Although this hypothesis has not been rigorously tested in *S. aureus* yet, our study and previous reports suggest that Isd receptors capture metalloporphyrins with high affinity, and transfer them to downstream Isd receptors.^{21,22} In addition, it has been proposed that the antimicrobial porphyrin Co(III)-PPIX blocks the transfer reaction from IsdA to IsdE via IsdC.²²

To improve the binding potency of these compounds, and possibly their antimicrobial properties, it is necessary to increase their affinity for the heme receptors beyond that of the natural ligand heme. Although this level of affinity has not been matched by any of the antimicrobial porphyrins tested so far by us in IsdH-NEAT3, our study unveils plausible routes toward achieving that goal. First, from our thermodynamic analysis (Figs. 6 and 7 and Table II) and a previous report²¹ we have concluded that only porphyrins bearing a metal in oxidation state of (III) are selected by the NEAT3 receptor. This observation limits the range of useful metal-porphyrins of high efficacy. Second, the affinity of metal(III)-porphyrin is

not greatly affected by the identity of the metal(III) ion, suggesting that gains of affinity can only be achieved by modification of the porphyrin ring. And third, the discovery that the NEAT3 receptor captures dimers of heme with minimum clashes with the protein reinforces the argument that the porphyrin ring moiety can be modified greatly without incurring in steric hindrance. Ga(III)-PPIX, being a metalloporphyrin exhibiting low toxicity to human fibroblast cells up to 100 $\mu\text{g mL}^{-1}$, and Balb/c mice up to 25–30 mg kg^{-1} , could be a suitable initial candidate for further development.²³ In conclusion, we hope these findings will be useful in the design of more effective antimicrobial compounds against *S. aureus*.

Materials and Methods

Metalloporphyrins

Cu(II)-PPIX, Zn(II)-PPIX, Mg(II)-PPIX, Mn(III)-PPIX, and Fe(III)-PPIX were purchased from Frontier Scientific (Logan, UT). Ga(III)-PPIX hydroxide was synthesized by the methodology described previously.⁴⁷ In(III)-PPIX was a gift from Prof. Nakayama (Nagasaki University).

Purification of apo-IsdH-NEAT3

Escherichia coli cells harboring the expression plasmid of IsdH-NEAT3 (residues Gly-534 to Gln-664) were grown in minimal M9 medium and purified as described previously.²¹ Briefly, cells were harvested, lysed by the sonication method, and the supernatant subjected to immobilized-metal affinity chromatography in a His-trap column (GE Healthcare, Piscataway, NJ). The His₆ tag was cleaved off with protease thrombin, and the excised peptide separated by rechromatography in the same His-trap column. Pure fractions (>95%) of IsdH-NEAT3 were obtained after size-exclusion chromatography in a HiLoad 16/60 superdex 200 column (GE Healthcare) equilibrated with 50 mM phosphate buffer.

Site-directed mutagenesis

Mutein Y642A was prepared with a Quick mutagenesis kit (Toyobo, Japan). Forward primer 5'-GCAAA CATTGGTGCTGAAGGTCAATATCATGTCAG-3' and reverse primer 5'-GCAAACATTGGTGCTGAAGGT CAATATCATGTC-3' were purchased from Operon. DNA was sequenced to ensure the mutation was incorporated correctly. Protein expression and purification was carried out as above.

Purification of apo-IsdA

The receptor IsdA was purified in the heme-permeable strain RP523 of *E. coli*. Expression and purification of apo-IsdA was carried as described elsewhere.²¹ Briefly, *E. coli* RP523 cells were grown anaerobically, induced with 1.0 mM IPTG, harvested by centrifugation, and disrupted by the sonication

method. The soluble fraction was purified by immobilized-metal affinity chromatography. The His₆-tag was cleaved off with thrombin, followed size-exclusion chromatography in a HiLoad 16/60 column equilibrated with 50 mM phosphate buffer at pH 7.5. The absence of the characteristic Soret band of heme molecule demonstrated that the purified IsdA corresponds to the apo-protein.

UV-visible absorbance spectroscopy

Freshly prepared In(III)-PPIX or Fe(III)-PPIX at a concentration of 10 μ M were titrated with apo-IsdH-NEAT3 in 50 mM phosphate buffer at pH 7.5. Spectra were promptly recorded within minutes between 250 and 650 nm at 50 nm min⁻¹ in a Jasco spectrophotometer (Tokyo, Japan) at 25°C. Three independent spectra were collected and averaged out for each heme:IsdH-NEAT3 ratio examined. We performed a second type of titration in which IsdH-NEAT3 (100 μ M) was incubated with heme o/n, followed by the removal of excess heme by ion-exchange chromatography as described previously.²¹

Transfer of In(III)-PPIX from IsdH-NEAT3 to apo-IsdA

Holo-IsdH-NEAT3 was prepared by incubation of In(III)-PPIX (or heme) with apo-receptor, followed by removal of excess porphyrin by ion-exchange chromatography. Holo-IsdH-NEAT3 was incubated with apo-IsdA and immediately separated by ion exchange chromatography on a DEAE column. The flow-through containing NEAT3 was analyzed by UV-visible spectroscopy in triplicate.^{13,21}

Crystallization of IsdH-NEAT3 in complex with In(III)-PPIX

Apo-IsdH-NEAT3 was mixed with excess In(III)-PPIX (~10 fold) for 1 h and subjected to ionic exchange chromatography in a DEAE column to remove the excess porphyrin. Single crystals of IsdH-NEAT3-In(III)-PPIX complex were obtained by the hanging drop vapor diffusion method by mixing the protein-ligand complex at a protein concentration of 11.5 mg mL⁻¹ with a precipitant solution composed of polyethylene glycol monomethyl ether 3500 (18–20% w/v), sodium iodide 0.2M, and potassium iodide 0.2M. Crystals developed in 4 months at 20°C to approximate dimensions of 0.05 mm $\times$ 0.05 mm $\times$ 0.03 mm. Suitable crystals were harvested, immersed for a few seconds in a cryoprotective solution composed of mother liquor supplemented with glycerol 20% (v/v), plunged in liquid N₂, and stored until data collection.

Data collection and structure refinement

Data collection under cryogenic conditions (100 K) was carried out at beamline AR-NE3A of the Photon

factory at Tsukuba (Japan). A single crystal of IsdH-NEAT3 with In(III)-bound diffracted to a resolution of 2.8 Å. The diffraction images were processed with the program MOSFLM⁴⁸ and merged and scaled with the program SCALA.⁴⁹ The structure was determined by the method of molecular replacement with the program PHASER⁵⁰ using the crystal structure of IsdH-NEAT3 with Ga(III)-PPIX bound (PDB entry code 3QUG).²¹ The crystallographic models were refined with the programs REFMAC⁵¹ of the CCP4 suite and COOT.⁵² The quality of the refined structure was assessed with the programs COOT and PROCHECK.⁵³ No outliers in the Ramachandran plot were observed.

Isothermal titration calorimetry

Thermodynamic parameters of the interaction between metalloporphyrins and IsdH-NEAT3 were determined with an iTC200 instrument (GE Healthcare) at 25°C. Heme (3.8 mg) was dissolved in 1.0 mL of DMSO to yield a concentration of 5 mM. Immediately before each titration, a 100- μ L aliquot of heme solution in DMSO was diluted in 9.9 mL of titration buffer (0.2M NaCl, 50 mM Tris-HCl, pH 8.0) to a final concentration of 50 μ M. Protein samples were equilibrated in titration buffer supplemented with 1% (v/v) DMSO. Aliquots containing IsdH-NEAT3 (250 μ M) were injected stepwise into the cell of the calorimeter containing the solution of freshly prepared heme (or metalloporphyrin) (50 μ M). The binding isotherms were fitted to a one-site binding model³⁰ using the program ORIGIN.

Molecular modeling

A model of IsdH-NEAT3 complexed with a molecule of (heme)₂ (IsdH-NEAT3-(heme)₂) was generated as follows. First, a model of Ga(III)-PPIX dimer (Ga(III)-PPIX)₂ was manually built using GaussView 5.0 based on a model previously reported.⁵⁴ The geometry was optimized at the PM6 semiempirical level using Gaussian 09.^{55,56} One porphyrin group of the optimized (Ga(III)-PPIX)₂ model was superimposed on the heme group of a crystal structure of IsdH-NEAT3 (PDB entry code: 2Z6F) with PyMOL.⁵⁷ The gallium atoms of (Ga(III)-PPIX)₂ were replaced with Fe(III). We note that the structure of (heme)₂ thus built is similar to that described in a previous computational study.⁵⁸

Prior to the molecular dynamics simulation, the N- and C-termini of IsdH-NEAT3 were capped with an acetyl group and an N-methyl group, respectively. Protonation states of the titratable residues were determined by PROPKA.⁵⁹ Water molecules were placed around the model using SOLVATE ver. 1.0.⁶⁰ Charge-neutralizing sodium ions (total of four ions) were also incorporated. The system was immersed in a water box using the LEAP module of AmberTools 1.5.⁶¹ The dimensions of the box were

determined so that the distance from the protein atoms to the closest boundary was at least 10 Å. The Amber ff99SB force field⁶² was used for IsdH-NEAT3, and the TIP3P model⁶³ was used for the water molecules. The parameters distributed with Amber 11 were used for the heme group. The parameters of the interaction between Tyr642 of IsdH-NEAT3 and heme, and between heme molecules, are given in supporting information Table S1. To calculate the atomic partial charges of heme and Tyr642, the complex between heme and 4-methylphenoxide was used as a model. The geometry of this complex was optimized at the B3LYP/6-31G(d) level with Gaussian 09 [14].⁵⁶ The atomic partial charges were calculated with the restrained electrostatic potential (RESP) method⁶⁴ using Antechamber.⁶⁵ The values of each atomic partial charge are given in supporting information Figure S4.

For the molecular dynamics simulation, the positions of the water molecules were optimized after a 200-step energy minimization, followed by a 200-step energy minimization of the entire system. The ensemble was gradually heated from 10 to 300 K, keeping the volume constant, over a period of 200 ps. The system was then equilibrated in a 800-ps constant-NPT simulation at 300 K and 1.0×10^5 Pa. Harmonic position restraints were imposed on non-hydrogen atoms of the IsdH-NEAT3-(heme)₂ complex and their force constants were gradually decreased from 41.8 to 0 kJ mol⁻¹ Å⁻² during the course of the simulations. The bond lengths involving hydrogen atoms were constrained by using the SHAKE algorithm,⁶⁶ which allowed the use of a time step of 2 fs. System temperature was controlled using Langevin dynamics⁶⁷ and system pressure was controlled using the weak coupling method.⁶⁸ The particle mesh Ewald method^{69,70} was used to calculate electrostatic interactions. All the MD simulations were conducted using the Sander or PMEMD module of Amber 11.⁶¹

Accession Codes

Coordinates and structure factors of IsdH-NEAT3 in complex with In(III)-PPIX have been deposited in the RCSB Protein Data Bank under accession code 3VTM.

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