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Editor's Choice

Mechanism of Heme Uptake by Heme Acquisition System A

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Heme acquisition system A (HasA), secreted by various gram-negative pathogens, uses a unique histidine–iron–tyrosine (His–Fe–Tyr) coordination to scavenge heme (i.e., protoporphyrin IX–Fe complex) as an iron source. In order to study heme uptake by HasA, we utilized axial ligand mutants and protoporphyrin IX. Our results suggest that the binding of heme in apoprotein is initiated by interactions between heme and hydrophobic residues in the heme pocket. Subsequently, the sequential coordination of Tyr-75 and His-32 with the iron appears to complete holoprotein formation.

Iron is involved in a wide range of essential functions in various metabolic pathways; therefore, most bacteria have some form of iron-scavenging system in order to survive.[1] Various gram-negative pathogens develop a heme acquisition system (Has) to use heme as a source of iron. An extracellular hemophore known as HasA captures free heme (i.e., protoporphyrin IX–Fe complex) and extracts heme from host hemoproteins. The heme bound to HasA is delivered to a specific outer membrane receptor known as HasR which translocates the heme into the periplasmic space.[2,3] The crystal structure of HasA from *Serretia marcescens* was determined in 1999,[4] and that of the corresponding protein from *Pseudomonas aeruginosa* was recently reported.[5–7] Heme is held by two loops in the heme-loaded holoprotein., The Nε atom of the histidine-32 (His-32) and the phenolate oxygen atom of the tyrosine-75 (Tyr-75) ligate the heme iron(III) (Figure 1). This unique His–Tyr pair is observed only in a few proteins, including chloroplast hemoglobin[8] and the reduced cytochrome *c* maturation protein CcmE.[9] In order to clarify the mechanism of heme uptake and release of heme by HasA, we expressed and purified HasA from *P. aeruginosa*. Subsequently, we attempted to explore the role of axial ligands in heme-transfer processes.

HasA from *P. aeruginosa* was expressed as a His-tag fusion protein in *Escherichia coli* and purified using a His-accept and an anion-exchange column. A single protein band corresponding to a molecular mass of 21 kDa was observed on the SDS-PAGE gel after purification. The absorption spectrum of the purified holo-HasA exhibited a Soret band at 407 nm and visible bands at 495, 540, 577, and 616 nm at 298 K (Supporting Information (SI), Figure S1a[10]) when the heme iron was in the Fe(III) state. We varied the pH values of holoprotein solution from 3 to 12, but no pH-dependent absorption spectral changes were observed. The electron spin resonance (ESR) spectrum of iron(III) holo-HasA obtained at 7 K showed major signals at $g = 2.78$, 2.19, and 1.75, suggesting the presence of low-spin six-coordinate heme iron ligated by His and Tyr (SI, Figure S1b[10]).

Attempts to reduce the heme iron(III) in the holoprotein by dithionite were unsuccessful. The absorption spectrum of holo-HasA solution containing dithionite was identical to that of iron(III) holoprotein. However, the addition of carbon monoxide (CO) gas to the solution shifted the equilibrium toward the heme iron(II), and the iron(II)–CO form of HasA with a Soret band at 413 nm was observed (SI, Figure S1a[10]). Since the CO–Fe(II)–His coordination typically shows a Soret band at 420 nm, CO gas appeared to replace His-32 to form the CO–Fe(II)–Tyr coordination in holo-HasA.[5,11]

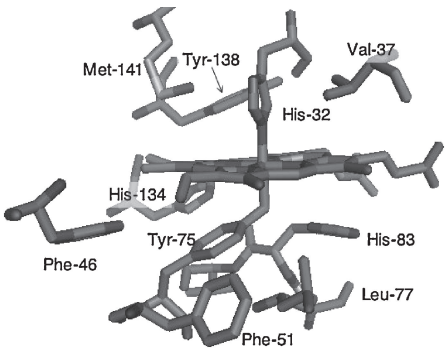

Figure 1. Heme binding site of HasA. His-32 and Tyr-75 are axial ligands for the heme iron(III). Some selected hydrophobic residues are also shown.

In order to study heme uptake, we prepared heme-free apoprotein by cold acid–acetone treatment and performed heme titration experiments. Free hemin solution exhibited an absorption maximum at 390 nm. Upon the addition of iron(III) hemin to apo-HasA, an increase in absorbance at 407 nm was observed. The absorbance reached a maximum at a heme to protein ratio of approximately one, indicating that holo-HasA retains one molecule of heme per molecule of protein. The absorption spectral changes upon the mixing of iron(III) heme and apo-HasA were monitored by stopped-flow spectrometry and fitted to the mechanism described in eq 1.

$$\text{apoprotein (colorless)} + \text{heme (colored)} \rightarrow \text{intermediate (colored)} \rightarrow \text{holoprotein (colored)} \quad (1)$$

The rate constants for the five-coordinate intermediate and the six-coordinate holoprotein formation were 150 ± 17 and $3.8 \pm 0.6\,s^{-1}$, respectively (Figure 2a). In contrast, no iron(II) heme was captured by apo-HasA. Surprisingly, protoporphyrin IX appeared to be bound by HasA even though no iron was present in the macrocycle. A broad Soret band of protoporphyrin IX at 380 nm was shifted to an intense peak at 407 nm upon the addition of apoprotein to protoporphyrin IX. The titration experiment suggested that one molecule of holoprotein held approximately one molecule of protoporphyrin IX (Figure 2b). Rapid scanning absorption spectral changes are consistent with the reaction mechanism described in eq 2,

$$\text{apoprotein (colorless)} + \text{protoporphyrin IX (colored)} \rightarrow \text{holoprotein (colored)} \quad (2)$$

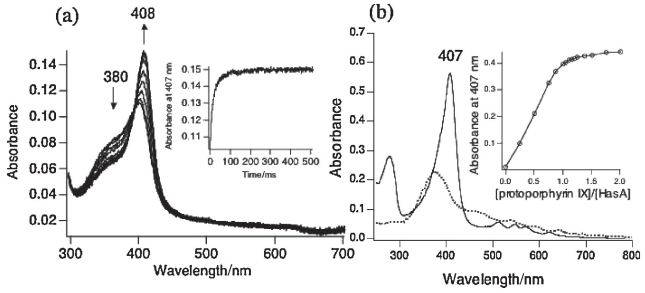

Figure 2. (a) Absorption spectral changes upon the mixing of protoporphyrin IX–Fe complex (i.e., heme) and wild type apo-HasA in phosphate buffered saline at 20 °C. Spectra at 1, 3, 7, 15, 31, 63, 127, and 255 ms after mixing. (b) Absorption spectra of free (dotted line) and bound form (solid line) of protoporphyrin IX in phosphate buffered saline. Protoporphyrin IX captured by wild type apo-HasA exhibited a Soret band at 407 nm. The inset indicted the results of protoporphyrin IX titration with wild type HasA.

and the rate constant was $77 \pm 4.0\,s^{-1}$. Thus, heme might enter the binding pocket of wild type HasA by hydrophobic interactions with phenylalanine, valine, and leucine in the binding site (Figure 1), and the subsequent coordination of axial ligands with the heme iron will generate the six-coordinate holoprotein.

We next constructed H32A and Y75A HasA to explore the sequence of axial ligations. The H32A mutant retained one molecule of heme per molecule of protein and exhibited a Soret band at 405 nm (SI, Figure S2a[10]). This value is similar to that of bovine liver catalase where the heme iron(III) is in the five-coordinate state with Tyr as the axial ligand.[11–13] Furthermore, high-spin signals appeared at $g = 6$ and 2 in the ESR spectrum at 7 K (SI, Figure S2b[10]). The addition of dithionite reduced the heme iron of the H32A mutant although the iron(II) form of the wild type was not observed under the same conditions. Soret bands for both the iron(II) and iron(II)–CO form of the H32A mutant appeared at 432 and 413 nm, respectively (SI, Figure S2a[10]). Absorption spectral changes for the iron(III) heme transfer to apo-H32A HasA are in good agreement with the kinetic model of apo-H32A (colorless) + heme (colored) → holo-H32A (colored), and the rate constant was $190 \pm 7.6\,s^{-1}$. This value is comparable to that of the intermediate formation for the wild type (i.e., $150 \pm 17\,s^{-1}$). Thus, the elimination of His-32, one of the axial ligands, did not diminish the ability for HasA to capture the heme.

We also found that the Y75A mutant bound heme. Soret bands of iron(III)– and iron(II)–CO Y75A HasA appeared at 406 and 419 nm, respectively, presumably due to the ligation of His-32 to iron in the mutant (SI, Figure S3[10]). Absorption spectral changes for the iron(III) heme transfer to apo-Y75A HasA were fitted to the mechanism of apo-Y75A (colorless) + heme (colored) → holo-Y75A (colored), and the rate constant was $5.6 \pm 0.3\,s^{-1}$, indicating that the replacement of Tyr-75 with alanine decelerated the heme transfer. This value was significantly smaller than that of the intermediate formation (i.e., $150 \pm 17\,s^{-1}$ for the first step of eq 1) but similar to that of the holoprotein formation (i.e., $3.8 \pm 0.6\,s^{-1}$ for the second step of eq 1) for the heme transfer by the wild type. The results of heme transfer by H32A and Y75A HasA suggest that Tyr-75 ligates faster than His-32 to the heme iron. Somewhat surprisingly, the H32A/Y75A double mutant could capture heme (SI, Figure S4[10]), and the heme-loaded double mutant showed the absorption spectrum similar to that of the wild type holoprotein. The possibility that His-83, His-134, and Tyr-138 serve as alternative ligands for the heme in the hydrophobic binding pocket is now under investigation.

In summary, we utilized axial ligand mutants and protoporphyrin IX to study heme uptake by HasA. Our observations revealed that the binding of heme in apoprotein might be initiated by interactions between heme and hydrophobic residues in the binding pocket. Subsequently, the sequential coordination of Tyr-75 and His-32 with the iron appears to complete the formation of holoprotein.

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