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Reaction Intermediates in the Heme Degradation Reaction by HutZ from *Vibrio cholerae*

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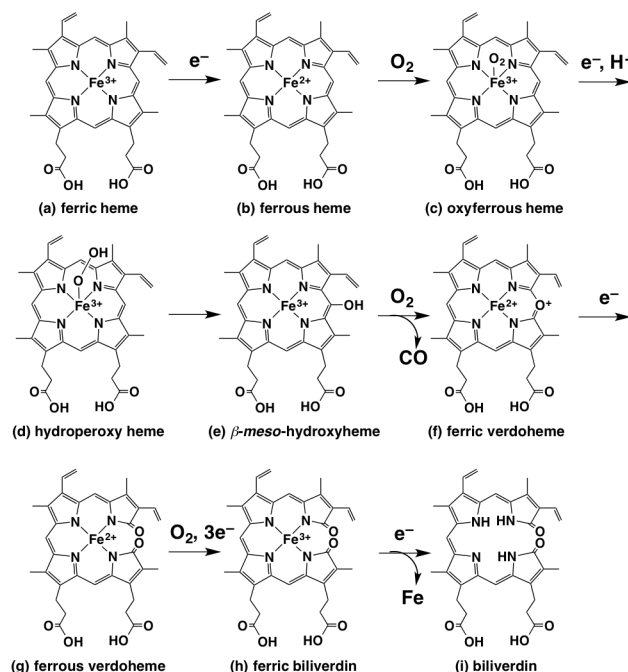
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HutZ is a heme-degrading enzyme in *Vibrio cholerae*. It converts heme to biliverdin via verdoheme, suggesting that it follows the same reaction mechanism as that of mammalian heme oxygenase. However, none of the key intermediates have been identified. In this study, we applied steady-state and time-resolved UV-vis absorption and resonance Raman spectroscopy to study the reaction of heme-HutZ complex with H₂O₂ or ascorbic acid. We characterized three intermediates: oxyferrous heme, *meso*-hydroxyheme, and verdoheme complexes. Our data support the view that HutZ degrades heme in a manner similar to mammalian heme oxygenase, despite their low sequence and structural homology.

1. Introduction

Iron is an essential element for bacterial growth, because it is contained in a group of proteins that play key roles in metabolic processes. Most of iron in the human body is present as heme iron. Therefore, heme is a dominant iron source for most pathogenic bacteria.¹ Heme-degrading enzymes in pathogens break heme and extract its iron, which is then utilized as a cofactor in other proteins.² In our previous study, we found that HutZ from *Vibrio cholerae* is capable of degrading heme when heme is in a high-spin state, but the reaction is quite slow when heme is in a low-spin state.³ We discovered that the heme degradation products of HutZ are verdoheme and biliverdin,³ as observed in the reaction catalyzed by mammalian heme oxygenase (HO),^{4–6} suggesting that HutZ follows the same reaction mechanism as that of HO (Scheme 1).⁷ In that mechanism, the initial step is the reduction of ferric heme to ferrous heme. Then, O₂ binds to the ferrous heme to generate oxyferrous heme (Fe²⁺-O₂). Further reduction and protonation of the oxyferrous heme leads to ferric hydroperoxy heme (Fe³⁺-OOH), which then reacts with the heme macrocycle to give *meso*-hydroxyheme. Subsequently, in the presence of O₂, the hydroxylated *meso*-carbon is eliminated as carbon monoxide (CO) with a concomitant formation of the verdoheme. The verdoheme is then oxygenated to generate biliverdin.



Scheme 1 Proposed heme degradation mechanism of HutZ.

The unusual self-oxygenation reactions of HO are achieved by its unique structure, namely a water cluster in its heme distal pocket (Fig. 1A). The water cluster of HO play critical roles in both *meso*-hydroxylation of heme and ring opening of verdoheme,^{8,9} whereas no such water molecules were found in the structure of a homologous protein, HugZ, from *Helicobacter pylori* (Fig. 1B).¹⁰ The water cluster is also absent in IsdG-type heme degrading enzymes, which produce distinct heme catabolites.^{11–13} Recently, an IsdG-type enzyme from *Mycobacterium tuberculosis* is shown to degrade heme by sequential mono- and dioxygenation reactions in contrast to

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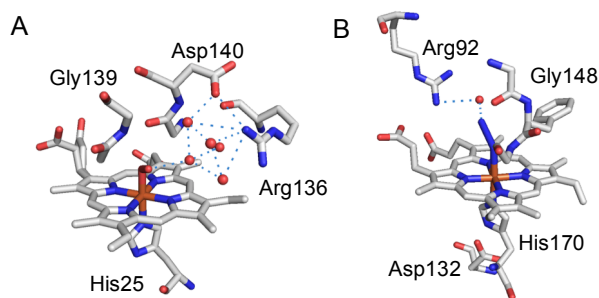


Fig. 1 Crystal structures of (A) rat HO-1 (PDB ID 4G7L) and (B) a HutZ homologous protein HugZ from *Helicobacter pylori* (3GAS). The residue numbering in (B) is based on *V. cholerae* HutZ. In HO-1, Asp140 forms a hydrogen bonding network with a water cluster, which is essential for the heme degradation activity of the enzyme. Arg92 in HugZ forms a hydrogen bond with iron-bound azide through a water molecule.

the canonical three monooxygenation mechanisms of HO.¹⁴ The mechanistic diversity prompted us to examine whether HutZ catalyzes the HO-type heme degradation in spite of their structural dissimilarity. In this study, we have characterized key reaction intermediates of HutZ, including oxyferrous heme, *meso*-hydroxyheme and verdoheme using UV-visible absorption and resonance Raman spectroscopy, thereby determining its heme-degradation mechanism.

2. Materials and methods

Materials

The chemicals used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma-Aldrich (St. Louis, MO, USA), and used without further purification. ¹⁸O₂ was purchased from Icon Isotopes (Summit, NJ, USA). Nitrogen and ¹⁶O₂ gas were purchased from Tech Air (White Plains, NY, USA).

Expression and Purification

HutZ was expressed in *Escherichia coli* and purified as described previously.³ Briefly, the *hutZ* gene was subcloned into pET-28b (Merck Millipore, Darmstadt, Germany) via *Nde*I and *Eco*RI sites, and the thrombin recognition site (Leu-Val-Pro-Arg-Gly-Ser) in the pET-28b construct was mutated to the HRV 3C protease recognition site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro).¹⁵ *E. coli* strains transduced with HutZ expression plasmids were grown at 37 °C in LB broth supplemented with 50 µg/mL kanamycin. Expression of His-tagged fusion protein in *E. coli* BL21(DE3) was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM, after which cells were further incubated at 28 °C overnight. His₆-tagged HutZ was purified by affinity chromatography using a HisTrap HP column (GE Healthcare, Uppsala, Sweden). After cleavage of the His₆-tag, the reaction mixture was applied to the HisTrap column. The column flow-through was collected and then applied to a gel-filtration column (HiLoad 16/60 Superdex 200 pg, GE Healthcare) equilibrated with 50 mM Tris-HCl and 150 mM NaCl (pH 8.0). The protein concentration was

determined on the basis of absorbance at 411 nm using extinction coefficient (ϵ_{412}) of 166 mM⁻¹cm⁻¹.¹⁶

Absorption Spectra Measurements

The reaction of HutZ with H₂O₂ was monitored by UV-visible absorption spectrophotometer (V-660, Jasco, Tokyo, Japan). Briefly, 1.9 mL of heme-HutZ solution (final concentration, 10 µM) in 50 mM Tris-HCl and 150 mM NaCl (pH 8.0) was placed in a cuvette, and the reaction was started by adding H₂O₂ in the same buffer at 25 °C. Spectra were recorded at 1-min intervals. Time-resolved UV-Vis absorption spectra were measured using stopped-flow apparatus (Applied Photophysics, Surrey, U.K.). The enzyme was rapidly mixed with O₂-containing buffer. The reaction kinetics were monitored using a photodiode array detector, and these data were analyzed with the ProK software from Applied Photophysics.

Resonance Raman Measurements

Time-resolved resonance Raman spectra were measured using a home-made continuous flow apparatus.¹⁷ Raman scattering was excited by the 413.1 nm line of a krypton ion laser (BeamLok 2080, Spectra Physics, Santa Clara, CA, USA), and collected into a Spex 1.25 m polychromator (Jobin Yvon, Edison, NJ, USA) equipped with a liquid nitrogen-cooled CCD detector (Model 1100 PB; Roper Scientific, Princeton, NJ, USA). The spectra were calibrated with the Raman bands of met myoglobin, for which the accurate band positions were determined by independent experiments using a spinning cell system and indene as the Raman frequency standard.

3. Results and discussion

Observation of oxy ferrous heme-HutZ

To generate the oxy ferrous species, we first reduced the ferric heme-HutZ complex anaerobically using sodium dithionite and removed the excess dithionite using a gel-filtration column in a glove box. We then exposed the reduced heme complex to O₂. Unexpectedly, only ferric heme, not oxyferrous heme, was observed owing to rapid autoxidation, which is plausibly partly due to strong electron donation from the proximal histidine, as discussed in our previous work.³ To explore the kinetics of the autoxidation process, we repeated the reaction with a stopped-flow system. Fig. 2A shows the absorption spectral changes obtained following the mixing of the ferrous heme-HutZ complex with an O₂-containing buffer. The spectra were analyzed with a three-species sequential kinetic model (A → B → C). The standard spectra of the three species obtained from global analysis of the data are shown in Fig. 2B. The spectrum of the species A matches that of ferrous heme-HutZ (Table 1). The spectrum of the species C is similar to that of ferric heme-HutZ, but it also contains weak bands at ~620 and ~670 nm, which are not present in the ferric heme,³ but encountered in the ferrous verdoheme as discussed later (Fig. 5, red). The species B could be assigned to the oxyferrous heme complex, as it has the Soret band at 416 nm and Q-bands at 542 and 577 nm, similar to those observed in hemoglobin (Hb)¹⁸ and HO.¹⁹

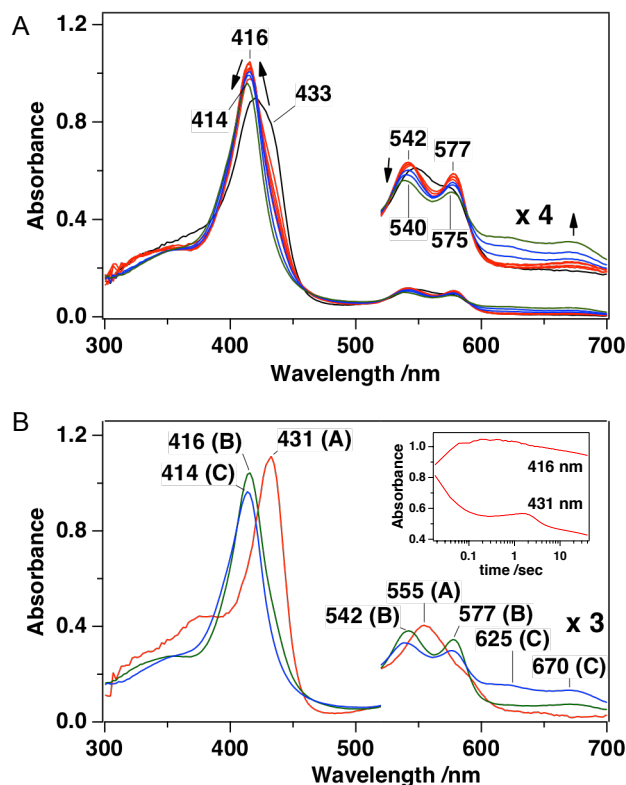


Fig. 2 (A) Absorption spectra obtained following the mixing of ferrous heme-HutZ with an O_2 -containing buffer. The spectra were taken at 0.018 (black), 0.039, 0.060, 0.081, 0.126, 0.204 (red), 2.12, 5.12 (blue), and 40 (green) sec after the initiation of the reaction. (B) Deconvoluted absorption spectra based on the three-species sequential kinetic model ($A \rightarrow B \rightarrow C$). Spectra of the species A, B, and C are shown in red, green, and blue, respectively. The inset shows the time courses of the absorbance changes at 416 and 431 nm.

The time evolutions of the oxyferrous and ferrous heme species as indicated by the absorbance changes at 416 and 431 nm, respectively, are shown in Fig. 2B. Based on the data, to characterize the oxyferrous heme species we measured the resonance Raman spectrum of HutZ at 350 ms following the initiation of the O_2 binding reaction, by using a homemade continuous-flow mixing device with a flow rate of 3 ml/min.¹⁷

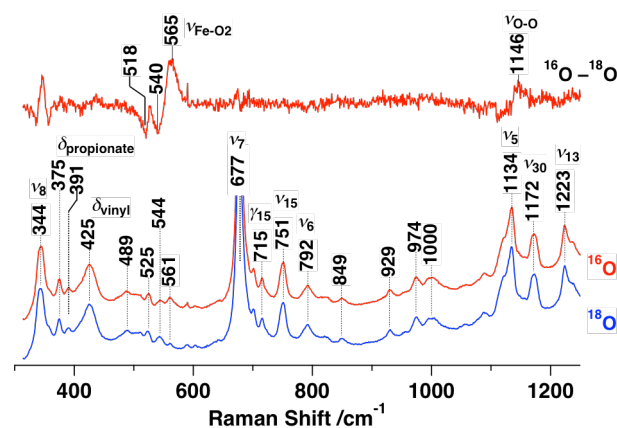
Table 1. Electronic absorption bands for deconvoluted species observed in the reaction of ferrous heme-HutZ with O_2 -containing buffer, and representative heme proteins.

Protein	State	Soret nm	Q-band nm	Reference
species A ^a	(ferrous)	431	555	this work
heme-HutZ	ferrous	431	557	3
species B ^a	(oxyferrous)	416	542, 577	this work
heme-HO	oxyferrous	410	539, 575	19
Human Hb ^b	oxyferrous	415	541, 577	18
SW Mb ^c	oxyferrous	418	543, 581	18
species C ^a	(ferric + VD ^d)	414	539, 576	this work
heme-HutZ	ferric	412	542, 577	3

^a Color corresponds to that of spectra in Fig. 2. ^b Hb, hemoglobin, ^c SW Mb, sperm whale myoglobin, ^d VD, verdoheme.

As shown in Fig. 3, the resonance Raman spectrum of the O_2 -bound HutZ is dominated by strong in-plane vibrational modes of the heme, as well as modes associated with the peripheral groups attached to it. The oxygen-associated modes are identified by carrying out the measurement with $^{16}O_2$ and $^{18}O_2$ isotopes. In the $^{16}O_2$ - $^{18}O_2$ difference spectrum, all the heme modes are cancelled out, the remaining positive and negative peaks are associated with the ^{16}O and ^{18}O -related modes, respectively. The positive peak at 565 cm^{-1} is assigned to the ν_{Fe-O_2} mode, similar to that reported for human HO²⁰ and other heme proteins with histidine as a proximal ligand.²¹ It is noteworthy that the negative components associated with the 565 cm^{-1} peak exhibit 2 minima at 518 and 540 cm^{-1} because of the coupling of the ν_{Fe-O_2} mode to the heme mode at 525 cm^{-1} , similar to that reported for the oxy-derivative of a globin model system²² and to the oxy-ternary complex of human indoleamine 2,3-dioxygenase.²³ A weak oxygen-sensitive mode at 1146 cm^{-1} is tentatively assigned to a ν_{O-O} stretching mode, similar to that of an O_2 -bound heme with a ferric-superoxide electronic configuration.⁹ This assignment remains to be confirmed as the corresponding negative peak expected at $\sim 1075\text{ cm}^{-1}$ is too weak to be identified.

The frequency of the ν_{Fe-O_2} mode reflects the environment of the heme iron-bound O_2 . The ν_{Fe-O_2} mode of human Hb was reported at 568 cm^{-1} .^{24,25} In contrast, that of *Chlamydomonas* Hb was identified at 554 cm^{-1} .²⁶ The significantly lower frequency observed in *Chlamydomonas* Hb is believed to be a result of the hydrogen bonding interactions between Gln84/Tyr63 and the proximal/terminal oxygen of the heme iron-bound O_2 , respectively, as supported by the observation that Q84G or Y63L mutation leads to the upshift of the ν_{Fe-O_2} frequency to 569 and 561 cm^{-1} , respectively.²⁶ The frequency of the ν_{Fe-O_2} mode of the heme-HutZ complex is similar to that of HO-1, human Hb and the Q84G or Y63L mutants of



Chlamydomonas Hb, indicating the presence of at least one hydrogen bond to the iron-bound O₂. Indeed, the structure of HugZ from *H. pylori* suggests that the conserved Arg92 acts as a hydrogen bond donor for the iron-bound O₂ (Fig. 2B).¹⁰ This hydrogen bond in HutZ plausibly contributes to the regioselectivity of heme degradation at β - or δ -*meso* carbon.³

Observation of *meso*-hydroxyheme-HutZ

We next try generating the *meso*-hydroxyheme by mixing the ferric heme-HutZ complex with one equivalent of H₂O₂ under an anaerobic condition.²⁷ An anaerobic condition was essential as O₂ converts *meso*-hydroxyheme to verdoheme in a non-enzymatic fashion (see Scheme 1).^{28,29} The H₂O₂ reaction was monitored by UV-vis absorption spectroscopy. As shown in Fig. 4, following the initiation of the reaction the Soret band at 405 nm decreased with time, which reached a plateau at ~5 min. The spectral change was associated with the appearance of a weak band at ~619 nm, similar to that of the *meso*-hydroxyheme of HO. It has been reported that the α -, β -, and δ -*meso*-hydroxyheme of HO have absorption maxima at 405 nm, with a relatively featureless absorption band in the visible region, while the γ -*meso*-hydroxyheme of HO has an absorption maximum at 413 nm.³⁰ Hence our current data support the view that the heme of HutZ is cleaved at β - or δ -*meso* carbon.³ Upon the exposure to O₂, the 405/619 nm species immediately converted to a new species with an intense absorption at 644 nm (Fig. 4, red), which we tentatively assign to the ferric verdoheme-HutZ complex.

Characterization of verdoheme-HutZ

To further confirm the assignment of the 644 nm species, redox dependence of the spectrum was investigated. First, the 644-nm species was produced by reacting the ferric heme-HutZ complex with H₂O₂ under an aerobic condition (Fig. 5, green). Then, remaining H₂O₂ was removed by catalase, followed by anaerobic addition of ascorbic acid. The maximum of the band shifted from 644 to 671 nm (Fig. 5, red), consistent with the formation of the ferrous β - (667-668 nm) or δ -verdoheme (663-664 nm).^{30,31} Further addition of CO to the ferrous verdoheme caused a blue shift of the band to 625 nm

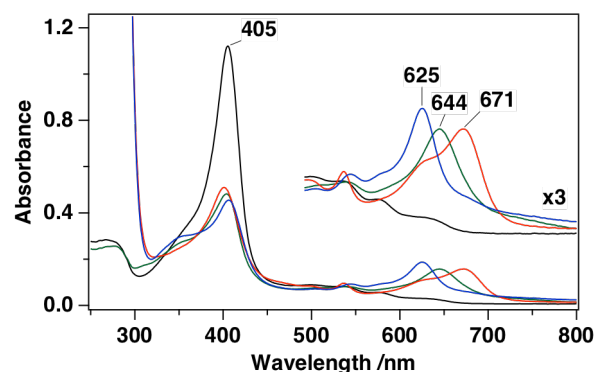


Fig. 5 Absorption spectra of verdoheme-HutZ complex. Black, ferric heme-HutZ; green, Fe³⁺-verdoheme generated by reaction of ferric heme with 1 equivalent of H₂O₂; red, Fe²⁺-verdoheme generated by reaction of Fe³⁺-verdoheme with 1 mM ascorbic acid; blue, CO-bound Fe²⁺-verdoheme generated by reaction of Fe²⁺-verdoheme with CO. Reaction was conducted under an anaerobic condition.

(Fig. 5, blue), in good agreement with the production of CO-bound β -verdoheme (623-624 nm) or δ -verdoheme (624-626 nm).^{30,31} These results confirm that the 644-nm species is a ferric verdoheme as typically observed for other HOs, not a ferrous verdoheme.

Lastly, we investigated the peroxidation activity of heme-HutZ using *o*-methoxyphenol (guaiacol) as a substrate (Fig. 6). The 470 nm absorption, which is derived from the oxidized guaiacol oligomer, showed a pronounced lag phase (<200 s) before a linear production phase was observed. During the first stage with negligible peroxidation, absorbance at 644-nm increases due to formation of ferric verdoheme from heme-HutZ. This observation strongly suggests highly efficient self-oxygenation of heme-HutZ to yield *meso*-hydroxyheme without generating ferryl heme. In fact, the anaerobic reaction with one mole equivalent of H₂O₂ (Fig. 4) yields approximately 70 % verdoheme after exposure to air as judged from the absorbance increase at 644-nm. At the latter stage of the peroxidation assay (>200 s), the 470-nm absorbance shows linear increase with small decrease in the 644-nm absorbance, suggesting efficient peroxidation by ferric verdoheme. It has been reported that the verdoheme complex of HO-1 is active towards H₂O₂ only in its ferrous state,³² because a water

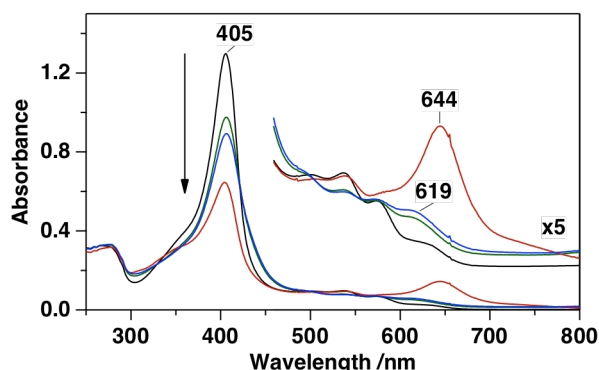


Fig. 4 Reaction of ferric heme-HutZ (10 μ M) with 1 equivalent of H₂O₂ at pH 6.0. Black, before the reaction; green and blue, 20 sec and 5 min after the reaction, respectively; red, 15 min after addition of air to the blue species. Reaction was conducted under an anaerobic condition.

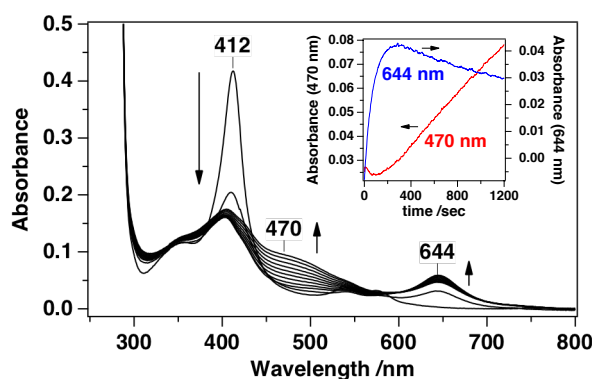


Fig. 6 Reaction of ferric heme-HutZ with H₂O₂ in the presence of 1 mM guaiacol. The inset shows the time courses of the absorbance at 470 and 644 nm. The spectra were measured at 2-min intervals for 30 min after addition of 0.1 mM H₂O₂.

molecule (or OH⁻), which is bound to ferric heme as the sixth ligand, inhibits the reaction with H₂O₂. To react with heme, H₂O₂ must replace the water molecule. Therefore, the low reactivity of ferric verdoheme-HO is plausibly ascribed to tight water ligation on the ferric iron, which inhibits the H₂O₂ binding. Accordingly, the unexpected peroxidation activity of ferric verdoheme-HutZ is possibly a result of a weaker water coordination.

Conclusions

We characterized three intermediates populated during the heme degradation reaction of HutZ from *V. cholerae*: oxyferrous heme, *meso*-hydroxyheme, and verdoheme. The $\nu_{\text{Fe-O}_2}$ for oxyferrous HutZ was the same as that of HO (Fig. 2).²⁰ HutZ is found to specifically convert the ferric heme into *meso*-hydroxyheme, at least in the reaction with H_2O_2 (Fig. 5), supporting that its active site is highly optimized as a heme-degrading enzyme. The observation that the reaction intermediates of HutZ are similar to those observed in the HO-1 reaction indicates that the heme degradation pathway of HutZ is the same as that of mammalian or canonical HOs, although an early experimental study reported that HutZ is a heme-binding protein, but not a heme-degrading enzyme.¹⁶ The fact that HutZ has no sequence and structural similarity to HOs (Fig. 7 and Fig. S1[†]) and IsdG (Fig. S2[†]) indicates that HutZ has evolved from ancestral distinct from that of HOs, although their heme degradation mechanism is conserved.

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HO-1      MERPQLDSMSQDLSEALKEATKEVHIRAENSEFMRFNQKQ
HutZ      -----

HO-1      QVSREGFKLVMASLYHIYTALEEEIERNKQNPVYAPLYFP
HutZ      -----MDQQVQKERLQGRLEP
              :::: :: *

HO-1      EELHRRAALEQDMAFWYGPHWQEAIPYTPATQHYVKRLHE
HutZ      EIKEFRQERKTLQLATVDAQGRPNVSYAPFVQNQEGYFVL
              * . * : . : : :.*.*.*: :

HO-1      VGGTHPELLVAHAYTRYLGDLGGQVLKKIAQKAMALPSS
HutZ      IS-----HIARHARN-----LEVNPQVSIMMIEDE
              :. :. ** . : : . * : ..

HO-1      GEGLAFFTFPSIDNPTKFKQLYARMNTLEMTPEVKHRVT
HutZ      TEAKQLFARKRLT-----FDAVASMVERDSELWCQVI
              *. :*: : : * . :* .*: :*

HO-1      EEAKTAFLNLNIELFEELQALLTEEHK-DQSPSQTEFLRQR
HutZ      AQMGERGEIIDGLSQLQDFMLFRLQPEQGLFVKGFQQAY
              : * *: :.:** : : . : :*. . * :

HO-1      PASLVQDTTSAETPRGKSQISTSSSQTP
HutZ      QVSGDDLVDVFVHLEEGHRKISNG-----
              * * * * *

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Fig. 7 Amino acid sequence comparison between HutZ and rat HO-1 using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Acknowledgements

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