Journal of Inorganic Biochemistry 259 (2024) 112654

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Short Communication

Heme *d* formation in a *Shewanella benthica* hemoglobin

Jaime E. Martinez Grundman [a,1], Thomas D. Schultz [a], Jamie L. Schlessman [b], Kevin Liu [a], Eric A. Johnson [c], Juliette T.J. Lecomte [a,*]

[a] *T.C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD 21218, USA*
[b] *Chemistry Department, U.S. Naval Academy, Annapolis, MD 21402, USA*
[c] *Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA*

ARTICLE INFO

Keywords:
Truncated hemoglobin
Heme modification
Extremophile
Heme *d*
Shewanella benthica
Heme adduct

ABSTRACT

In our continued investigations of microbial globins, we solved the structure of a truncated hemoglobin from *Shewanella benthica*, an obligate psychropiezophilic bacterium. The distal side of the heme active site is lined mostly with hydrophobic residues, with the exception of a tyrosine, Tyr34 (CD1) and a histidine, His24 (B13). We found that purified SbHbN, when crystallized in the ferric form with polyethylene glycol as precipitant, turned into a green color over weeks. The electron density obtained from the green crystals accommodated a *trans* heme *d*, a chlorin-type derivative featuring a γ-spirolactone and a vicinal hydroxyl group on a pyrroline ring. In solution, exposure of the protein to one equivalent of hydrogen peroxide resulted in a similar green color change, but caused by the formation of multiple products. These were oxidation species released on protein denaturation, likely including heme *d*, and a species with heme covalently attached to the polypeptide. The Tyr34Phe replacement prevented the formation of both heme *d* and the covalent linkage. The ready modification of heme *b* by SbHbN expands the range of chemistries supported by the globin fold and offers a route to a novel heme cofactor.

Hemoglobin proteins are used by organisms in all three domains of life [1]. This broad distribution is accompanied with functions adapted to specific metabolic needs and environmental conditions [2,3]. Naturally, trends in reactivity have been correlated against available structural information across the superfamily with the goal to predict which enzymatic or binding function dominates in any given globin. For example, ligand affinity is strongly related to the amino acid makeup of the heme pocket [4,5]. To refine these reactivity trends and deepen our understanding of globin chemical versatility, we initiated the study of a group 1 truncated hemoglobin belonging to an incompletely characterized phylogenetic clade known as subgroup 2 [6]. Our target is from the deep-sea proteobacterium *Shewanella benthica* strain KT99, a facultative anaerobe that thrives at cold temperatures and high hydrostatic pressures [7–9]. In a series of experiments probing the recombinantly expressed globin for potential activity, we considered the effect of peroxides. Here we describe the unexpected formation of *trans* heme *d* and draw a distinction between peroxide reactivity in solution and in the crystalline state.

The primary structure of SbHbN (Supplementary Material Fig. S1) has cysteines at positions 51 and 71 (in the myoglobin nomenclature, these positions are E16 and F10, respectively). Cys51(E16) and Cys71 (F10) are predicted to be outside of the heme pocket and not within contact of each other. We replaced these cysteines with serines to eliminate confounding redox reactions. The recombinant pseudo wild-type protein, termed S2SbHbN hereafter, was prepared heterologously in *E. coli* and purified in a stable ferric form using a standard protocol (Supplementary Material and [10]). Intentional exposure of ferric S2SbHbN in solution to one equivalent of H_2O_2 resulted in a rapid discoloration of the solution (Fig. 1). When we monitored reaction progress by electronic absorption spectroscopy, we noted that the Soret absorbance intensity dropped during the 6-s dead time. A band appeared at 675 nm then decayed slowly ($t_{1/2}$–5 min), while absorbance at ~595 nm increased and plateaued. Treatment of the peroxide-reacted protein by acidification and butanone extraction [11], a standard procedure for

Abbreviations: Hb, hemoglobin; HbN, truncated hemoglobin group N; S2SbHbN, Group 1 truncated hemoglobin (Uniprot A9DF82) with Cys51Ser and Cys71Ser replacements; *S. benthica*, *Shewanella benthica* strain KT99; Sb, *Shewanella benthica*.

* Corresponding author at: Department of Biophysics, Johns Hopkins University, 3400 North Charles Street, Baltimore, MD 21218, USA.
E-mail address: lecomte_jtj@jhu.edu (J.T.J. Lecomte).
[1] Present address: Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts, 02138, USA

https://doi.org/10.1016/j.jinorgbio.2024.112654
Received 11 May 2024; Received in revised form 25 June 2024; Accepted 25 June 2024
Available online 27 June 2024

heme removal, left most of the color in the aqueous phase.

Intact protein ultra-performance liquid chromatography mass spectrometry of a peroxide-reacted S2SbHbN sample (pH 7.0) revealed a mixture of two main polypeptide species: the intact apoprotein (12,823.3 Da) and a product with a molecular weight of 13,436.7 Da (Fig. S2) in roughly equal proportions. The latter is larger than that of the apoprotein by 613 Da, approximately 2–3 Da short of heme *b*. The higher mass confirmed covalent attachment of the heme group to the polypeptide, an adduct we refer to below as S2SbHbN*.

The mass spectrometry data also provided information on heme species not covalently attached to the protein. Mass signatures of unmodified heme *b* ($m/z = 616.2$), heme *b* plus one O atom ($m/z = 632.2$), and biliverdin-H^+ ($m/z = 583.7$) were readily detected (Fig. S3). The yield of each species depended on pH over the range 5.5–8.5; low pH favored S2SbHbN* and the oxidized heme, and high pH favored S2SbHbN and biliverdin (Fig. S3). Reaction of S2SbHbN with more than 1 equivalent of H_2O_2 resulted in additional modification(s) of the protein and heme degradation (Fig. S4), a common observation (e.g., [12]) due in part to the ability of the initial adduct to react with H_2O_2 and produce highly reactive hydroxyl radicals.

Formation of a green heme–protein adduct upon the action of H_2O_2 is reminiscent of the behavior of ferric myoglobin [13,14], in which protein radicals, notably a tyrosine, are involved [14–18]. In S2SbHbN, a candidate for a similar role is Tyr34(CD1) (Fig. 1F). A precedent for reaction at this site was reported by Yan and coworkers who characterized the Phe43(CD1)Tyr variant of myoglobin [19]. In contrast to the behavior of S2SbHbN, exposure of Y34F S2SbHbN to an equivalent amount of H_2O_2 caused reversible spectral changes (Fig. 1D) and no covalent attachment.

Covalent modification of heme proteins, and especially those involving radical species, are generally complex processes requiring extensive experimental work to achieve detailed structural identification and complete mechanistic description [18,20]. To begin the investigation of the S2SbHbN reaction, we sought to determine the structure of the unreacted protein. We observed that when samples of ferric S2SbHbN with added cyanide as an exogenous heme ligand were allowed to crystallize in the presence of polyethylene glycol under ambient light, crystals formed that, over time, changed color from red to brown to green, indicating loss of the cyanide ligand followed by modification of the heme group. No such transformation was observed in crystals of the Y34F variant. Different X-ray diffraction data sets were thus collected on cryogenically preserved S2SbHbN crystals at different stages of coloration (Table S1). One data set obtained on a red crystal resulted in a 1.8-Å model with the expected group 1 truncated hemoglobin fold topology and heme iron bearing cyanide (PDB 8UGZ, Fig. 1F). A second data set collected on a green crystal yielded a 2.0-Å model (PDB ID 8VSH) with two noteworthy features. First and surprisingly, Tyr34(CD1) and all other residues were unmodified compared to the structure of the red protein. The position of Tyr34(CD1) above the heme plane on the distal side was identical to that in the unreacted protein next to His24(B13) (Fig. 1F), the residue likely responsible for the pH dependence of product formation in solution. Second, density extending from ring D of the cofactor did not accommodate heme *b* substituents, i.e., a methyl group at C2D and a propionate at C3D (Fig. 1F). Instead, the density housed an additional hydroxyl group on C2D and a γ-spirolactone at C3D (Fig. 2). The carboxyl oxygen of the lactone points unambiguously to the distal side of the heme cavity. Electron density at C2D appears more intense in *trans* with respect to the ester than in *cis*. Thus, we built the modified heme with *R* stereocenters, distinct from the configuration assigned to the *d*-type heme in catalase

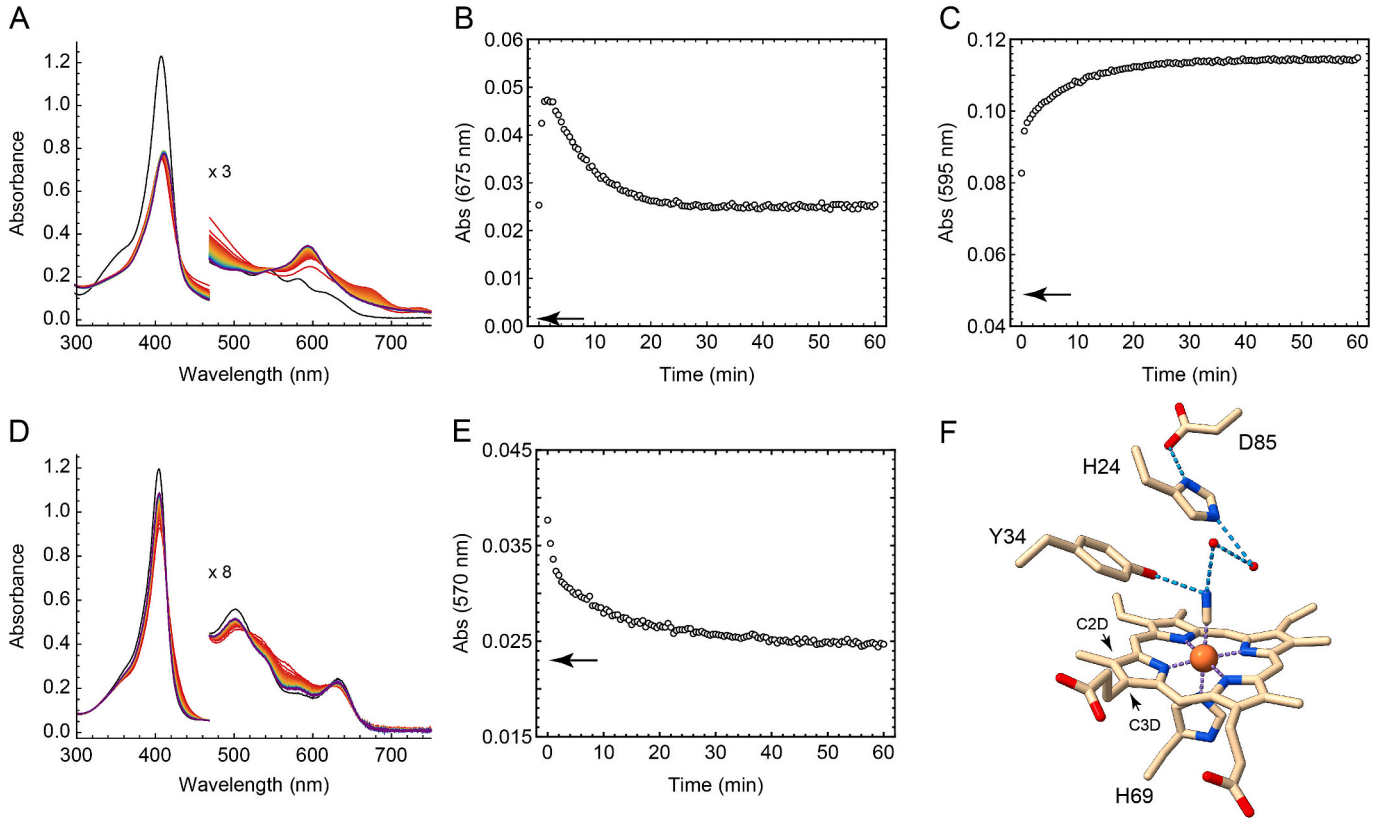


Fig. 1. Reaction of ferric S2SbHbN (9 μM, A–C) and its Y34F variant (7 μM, D–E) with equimolar amounts of H_2O_2 as monitored by electronic absorption spectroscopy. Black spectra: unreacted proteins; red to blue spectra: time course. The arrows in B, C, and E indicate the absorbance value of the ferric species prior to H_2O_2 addition. Conditions were 20 mM phosphate buffer, pH 7. Pre-treatment with cyanide inhibited the reaction. (F) Key residues in the distal pocket of cyanide-bound S2SbHbN (PDB ID 8UGZ, chain D, Table S1). Two carbons of interest on pyrrole D are indicated by arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

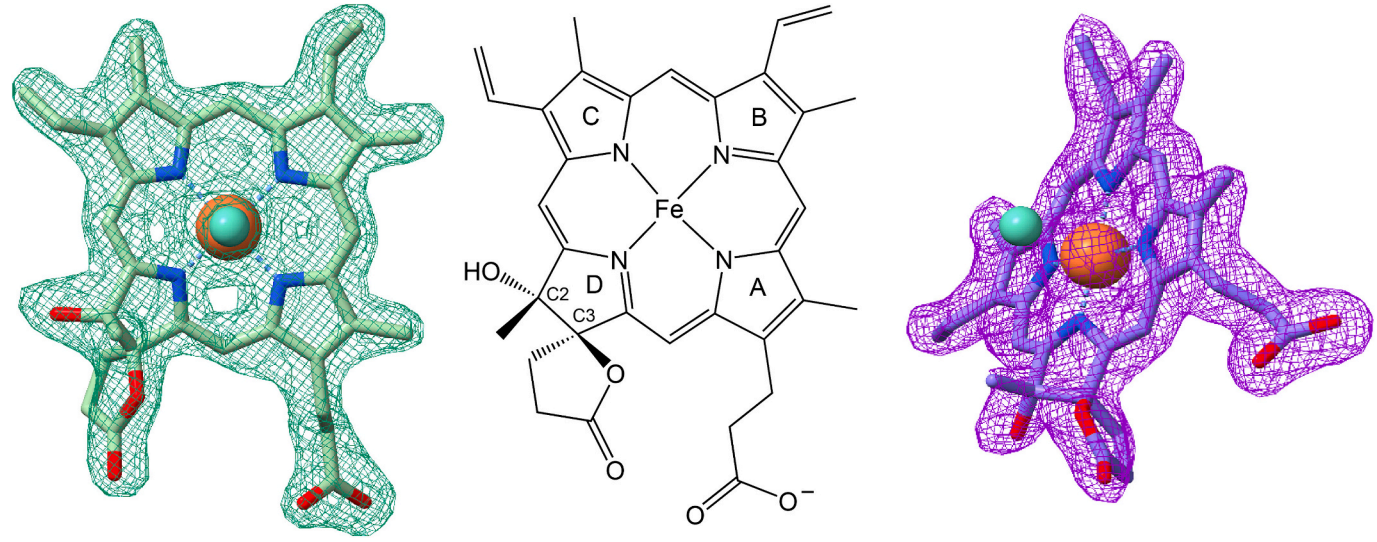


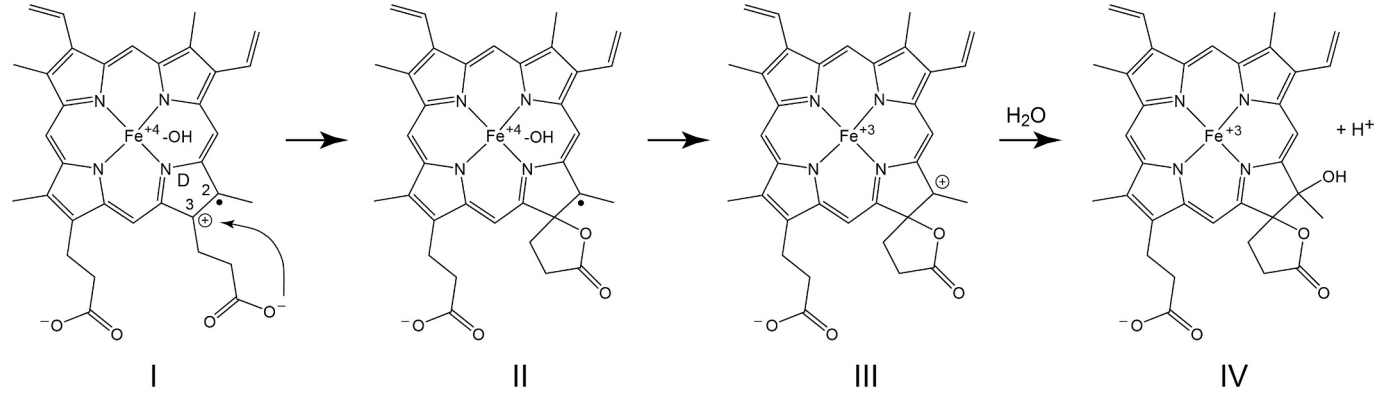
Fig. 2. Electron density ($2mF_o - DF_c$) of *trans* heme *d* in PDB structure 8VSH (green crystal). Left: chain C contoured at 1.2 σ. Right: chain A contoured at 1.0 σ emphasizing the differential density on the C2D substituents. The distal water molecule is shown with a cyan sphere to orient the view. The absolute configuration at C2 and C3 on pyrroline D is *R*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HPII [21–23] and cytochrome *bd* [24]. Electron density for the same modified heme was observed in all four chains in the asymmetric unit. Lastly, a third data set, from a crystal that had turned partially from red to green, was modeled to 1.8 Å with a mixture of heme *b* and *trans* heme *d* in each of the four chains in the asymmetric unit (PDB ID 8W3A) without the need for additional cofactor derivatives. The polypeptide chains of the three structures matched with root mean square deviations better than 0.2 Å. Overall, the data are consistent with the formation of unhydrolyzed heme *d* and minimal consequences for the protein structure, in agreement with a reaction that occurred in the crystal without damaging it.

The oxidative pathway proposed by Sugiyama et al. for the peroxide-induced reaction in myoglobin (Scheme 1, [25]) is plausible for SbHbN in its crystalline form: (i) reaction of the iron center with diffusing peroxide released by polyethylene glycol [26] and formation of a porphyrin cation radical; (ii) attack of the cationic site by the heme carboxylate on ring D and formation of a γ-spirolactone; (iii) return to the Fe(III) state; and (iv) addition of a water molecule and formation of an alcohol vicinal to the lactone. In this mechanism, differential salt bridge and steric interactions control the regio- and stereospecificity of the product. In SbHbN, the more stable *trans* product is favored, as originally proposed for cytochrome *bd* oxidase [27,28].

Returning to the reaction in solution, the species with $m/z = 632.2$ (heme plus one O atom) is tentatively identified as heme *d* because of the facile reaction in the crystal. The time series shown in Fig. 1A reflects the evolution of a complex mixture producing and consuming intermediates (e.g., 675 nm) and generating among other species a heme–protein cross-link (595 nm). It is likely that, when crystal packing interactions are absent and one equivalent of H_2O_2 is added at once, a radical is formed that migrates to a protein residue [25] (Scheme 1). The behavior of Tyr34Phe S2SbHbN suggests that Tyr34(CD1) plays a key role in the initial step, perhaps stabilizing species I in Scheme 1. We envision the next step to be an electron transfer from the porphyrin to a nearby residue such as Tyr60 in the EF loop or Tyr79(G5).

In catalases, the in-situ formation of *cis* heme *d* [29] may result from the action of singlet oxygen [30] via an epoxide intermediate [31]. In cytochrome *bd* oxidases, available structures also favor the *cis* isomer [24]. No dedicated enzyme has been found for the conversion, and self-modification is likely, either in the assembled cytochrome or in CydCD, the ABC transporter that delivers hemes to the periplasm [32–34]. The ease with which both *trans* heme *d* and a protein–heme covalent cross-link are formed in the *S. benthica* hemoglobin casts this protein as a test case for future mechanistic studies. Aspects such as the competition between conversion to heme *d* and cross-linking, control of heme *d* configuration and regiospecificity, effect on redox potential, role of cysteines, etc. can be addressed in this small protein. The modification may also signal different physiological roles related to reactive oxygen species management and the metabolism of heme in *S. benthica* and



Scheme 1. A possible path to heme *d* in the crystal, starting with a His–Fe(IV)–OH species. In solution, a competing process moves the radical from species I to a protein residue, leading to a protein–heme linkage. Adapted from [25].

suggest a path for the synthesis of *trans* heme *d* in this and related bacterial organisms.

CRediT authorship contribution statement

Jaime E. Martinez Grundman: Writing – review & editing, Writing – original draft, Investigation. **Thomas D. Schultz:** Investigation. **Jamie L. Schlessman:** Writing – review & editing, Methodology. **Kevin Liu:** Investigation. **Eric A. Johnson:** Writing – review & editing. **Juliette T. J. Lecomte:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Cited structures are available at the Protein Data Bank, entries 8UGZ, 8VSH, 8W3A.

Acknowledgements

We thank Dr. Maxime Siegler for X-ray data collection and processing and Dr. Phil Mortimer for assistance with the mass spectrometry experiments. This work was supported by the National Science Foundation grants CHE-2003950 to JTJL and GRFP-1746891 to JEMG. The X-ray diffractometer was purchased in part with funds from the National Institutes of Health, grant 1S10OD030352-01A1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2024.112654.

References

[1] S.N. Vinogradov, M. Tinajero-Trejo, R.K. Poole, D. Hoogewijs, Bacterial and archaeal globins — a revised perspective, Biochim. Biophys. Acta 2013 (1834) 1789–1800, https://doi.org/10.1016/j.bbapap.2013.03.021.
[2] M. Nardini, A. Pesce, M. Bolognesi, Truncated (2/2) hemoglobin: unconventional structures and functional roles in vivo and in human pathogenesis, Mol. Asp. Med. 84 (2022) 101049, https://doi.org/10.1016/j.mam.2021.101049.
[3] S.N. Vinogradov, L. Moens, Diversity of globin function: enzymatic, transport, storage, and sensing, J. Biol. Chem. 283 (2008) 8773–8777, https://doi.org/10.1074/jbc.R700029200.
[4] J.P. Bustamante, L. Radusky, L. Boechi, D.A. Estrin, A. ten Have, M.A. Martí, Evolutionary and functional relationships in the truncated hemoglobin family, PLoS Comput. Biol. 12 (2016) 26, https://doi.org/10.1371/journal.pcbi.1004701.
[5] C.D. Schuster, F. Salvatore, L. Moens, M.A. Martí, Globin phylogeny, evolution and function, the newest update, Proteins 2024 (2024) 1–15, https://doi.org/10.1002/prot.26659.
[6] D.A. Vuletich, J.T.J. Lecomte, A phylogenetic and structural analysis of truncated hemoglobins, J. Mol. Evol. 62 (2006) 196–210, https://doi.org/10.1007/s00239-005-0077-4.
[7] C. Kato, T. Sato, K. Horikoshi, Isolation and properties of barophilic and barotolerant bacteria from deep-sea mud samples, Biodivers. Conserv. 4 (1995) 1–9, https://doi.org/10.1007/BF00115311.
[8] C. Kato, L. Li, Y. Nogi, Y. Nakamura, J. Tamaoka, K. Horikoshi, Extremely barophilic bacteria isolated from the Mariana trench, challenger deep, at a depth of 11,000 meters, Appl. Environ. Microbiol. 64 (1998) 1510–1513, https://doi.org/10.1128/AEM.64.4.1510-1513.1998.
[9] F.M. Lauro, R.A. Chastain, S. Ferriera, J. Johnson, A.A. Yayanos, D.H. Bartlett, Draft genome sequence of the deep-sea bacterium *Shewanella benthica* strain KT99, Genome Announc. 1 (2013), https://doi.org/10.1128/genomea.00210-13.
[10] E.A. Johnson, S.L. Rice, M.R. Preimesberger, D.B. Nye, L. Gilevicius, B.B. Wenke, J. M. Brown, G.B. Witman, J.T.J. Lecomte, Characterization of THB1, a *Chlamydomonas reinhardtii* truncated hemoglobin: linkage to nitrogen metabolism and identification of lysine as the distal heme ligand, Biochemistry 53 (2014) 4573–4589, https://doi.org/10.1021/bi5005206.
[11] F.W.J. Teale, Cleavage of heme-protein link by acid methylethylketone, Biochim. Biophys. Acta 35 (1959) 543, https://doi.org/10.1016/0006-3002(59)90407-x.
[12] Y. Osawa, K. Korzekwa, Oxidative modification by low levels of HOOH can transform myoglobin to an oxidase, Proc. Natl. Acad. Sci. USA 88 (1991) 7081–7085, https://doi.org/10.1073/pnas.88.16.7081.
[13] J.B. Fox Jr., R.A. Nicholas, S.A. Ackerman, C.E. Swift, Multiple wavelength analysis of the reaction between hydrogen peroxide and metmyoglobin, Biochemistry 13 (1974) 5178–5186, https://doi.org/10.1021/bi00722a020.
[14] C.E. Catalano, Y.S. Choe, P.R. Ortiz de Montellano, Reactions of the protein radical in peroxide-treated myoglobin. Formation of a heme-protein cross-link, J. Biol. Chem. 264 (1989) 10534–10541, https://doi.org/10.1016/S0021-9258(18)81654-4.
[15] A. Wilks, P.R. Ortiz De Montellano, Intramolecular translocation of the protein radical formed in the reaction of recombinant sperm whale myoglobin with H_2O_2, J. Biol. Chem. 267 (1992) 8827–8833, https://doi.org/10.1016/S0021-9258(19)50354-4.
[16] B.J. Reeder, D.A. Svistunenko, M.A. Sharpe, M.T. Wilson, Characteristics and mechanism of formation of peroxide-induced heme to protein cross-linking in myoglobin, Biochemistry 41 (2002) 367–375, https://doi.org/10.1021/bi011335b.
[17] B.J. Reeder, F. Cutruzzola, M.G. Bigotti, N.J. Watmough, M.T. Wilson, Histidine and not tyrosine is required for the peroxide-induced formation of haem to protein cross-linked myoglobin, IUBMB Life 59 (2007) 477–489, https://doi.org/10.1080/15216540601178083.
[18] D.A. Svistunenko, Reaction of haem containing proteins and enzymes with hydroperoxides: the radical view, Biochim. Biophys. Acta 1707 (2005) 127–155, https://doi.org/10.1016/j.bbabio.2005.01.004.
[19] D.-J. Yan, W. Li, Y. Xiang, G.-B. Wen, Y.-W. Lin, X. Tan, A novel tyrosine–heme C–O covalent linkage in F43Y myoglobin: A new post-translational modification of heme proteins, ChemBioChem 16 (2015) 47–50, https://doi.org/10.1002/cbic.201402504.
[20] Y.-W. Lin, The broad diversity of heme-protein cross-links: an overview, Biochim. Biophys. Acta 2015 (1854) 844–859, https://doi.org/10.1016/j.bbapap.2015.04.019.
[21] G.N. Murshudov, A.I. Grebenko, V. Barynin, Z. Dauter, K.S. Wilson, B. K. Vainshtein, W. Melik-Adamyan, J. Bravo, J.M. Ferrán, J.C. Ferrer, J. Switala, P. C. Loewen, I. Fita, Structure of the heme *d* of *Penicillium vitale* and *Escherichia coli* catalases, J. Biol. Chem. 271 (1996) 8863–8868, https://doi.org/10.1074/jbc.271.15.8863.
[22] W. Melik-Adamyan, J. Bravo, X. Carpena, J. Switala, M.J. Maté, I. Fita, P. C. Loewen, Substrate flow in catalases deduced from the crystal structures of active site variants of HPII from *Escherichia coli*, Proteins 44 (2001) 270–281, https://doi.org/10.1002/prot.1092.
[23] A. Díaz, P.C. Loewen, I. Fita, X. Carpena, Thirty years of heme catalases structural biology, Arch. Biochem. Biophys. 525 (2012) 102–110, https://doi.org/10.1016/j.abb.2011.12.011.
[24] S. Safarian, C. Rajendran, H. Müller, J. Preu, J.D. Langer, S. Ovchinnikov, T. Hirose, T. Kusumoto, J. Sakamoto, H. Michel, Structure of a *bd* oxidase indicates similar mechanisms for membrane-integrated oxygen reductases, Science 352 (2016) 583–586, https://doi.org/10.1126/science.aaf2477.
[25] K. Sugiyama, R.J. Highet, A. Woods, R.J. Cotter, Y. Osawa, Hydrogen peroxide-mediated alteration of the heme prosthetic group of metmyoglobin to an iron chlorin product: evidence for a novel oxidative pathway, Proc. Natl. Acad. Sci. USA 94 (1997) 796–801, https://doi.org/10.1073/pnas.94.3.796.
[26] W.R. Wasylaschuk, P.A. Harmon, G. Wagner, A.B. Harman, A.C. Templeton, H. Xu, R.A. Reed, Evaluation of hydroperoxides in common pharmaceutical excipients, J. Pharm. Sci. 96 (2007) 106–116, https://doi.org/10.1002/jps.20726.
[27] R. Timkovich, M.S. Cork, R.B. Gennis, P.Y. Johnson, Proposed structure of heme *d*, a prosthetic group of bacterial terminal oxidases, J. Am. Chem. Soc. 107 (1985) 6069–6075, https://doi.org/10.1021/ja00307a041.
[28] M.R. Vavra, R. Timkovich, F. Yap, R.B. Gennis, Spectroscopic studies on heme *d* in the visible and infrared, Arch. Biochem. Biophys. 250 (1986) 461–468, https://doi.org/10.1016/0003-9861(86)90750-2.
[29] P.C. Loewen, J. Switala, I. von Ossowski, A. Hillar, A. Christie, B. Tattrie, P. Nicholls, Catalase HPII of *Escherichia coli* catalyzes the conversion of protoheme to *cis*-heme *d*, Biochemistry 32 (1993) 10159–10164, https://doi.org/10.1021/bi00089a035.
[30] W. Hansberg, Monofunctional heme-catalases, Antioxidants 11 (2022), https://doi.org/10.3390/antiox11112173.
[31] R. Timkovich, L. Bondoc, Diversity in the structure of hemes, Adv. Biophys. Chem. 1 (1990) 203–247.
[32] D. Wu, A.R. Mehdipour, F. Finke, H.G. Goojani, R.R. Groh, T.N. Grund, T.M. B. Reichhart, R. Zimmermann, S. Welsch, D. Bald, M. Shepherd, G. Hummer, S. Safarian, Dissecting the conformational complexity and mechanism of a bacterial heme transporter, Nat. Chem. Biol. 19 (2023) 992–1003, https://doi.org/10.1038/s41589-023-01314-5.
[33] C. Zhu, Y. Shi, J. Yu, W. Zhao, L. Li, J. Liang, X. Yang, B. Zhang, Y. Zhao, Y. Gao, X. Chen, X. Yang, L. Zhang, L.W. Guddat, L. Liu, H. Yang, Z. Rao, J. Li, Cryo-EM structures of a prokaryotic heme transporter CydDC, Protein, Cell 14 (2023) 919–923, https://doi.org/10.1093/procel/pwad022.
[34] R.K. Poole, A.G. Cozens, M. Shepherd, The CydDC family of transporters, Res. Microbiol. 170 (2019) 407–416, https://doi.org/10.1016/j.resmic.2019.06.003.