The Crystal Structure of Synechocystis Hemoglobin with a Covalent Heme Linkage*

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The x-ray crystal structure of Synechocystis hemoglobin has been solved to a resolution of 1.8 Å. The conformation of this structure is surprisingly different from that of the previously reported solution structure, probably due in part to a covalent linkage between the heme 2-vinyl and His117 that is present in the crystal structure but not in the structure solved by NMR. Synechocystis hemoglobin is a hexacoordinate hemoglobin in which the heme iron is coordinated by both the proximal and distal histidines. It is also a member of the “truncated hemoglobin” family that is much shorter in primary structure than vertebrate and plant hemoglobins. In contrast to other truncated hemoglobins, the crystal structure of Synechocystis hemoglobin displays no “ligand tunnel” and shows that several important amino acid side chains extrude into the solvent instead of residing inside the heme pocket. The stereochemistry of hexacoordination is compared with other hexacoordinate hemoglobin and cytochromes in an effort to illuminate factors contributing to ligand affinity in hexacoordinate hemoglobins.

A wide diversity in both form and function has been discovered in the study of hemoglobins (Hbs)† from many species, including bacteria (1, 2), plants (3), and humans (4–7). Although the physiological functions of many hemoglobins are still unknown, a number of new functions have recently been described, including the scavenging of nitric oxide and oxygen (2, 8), aerotaxis (9), and phototaxis (10). Although functions vary, the tertiary structures of these hemoglobins conserve many of the general features of the globin fold even when truncated to very short primary structures (11–13). However, like many proteins sharing the same fold but carrying out different functions, the specific amino acid residues surrounding the heme prosthetic group exhibit a large degree of variability across hemoglobins from different species.

An extreme example of this diversity is found in the family of hexacoordinate hemoglobins (hxHbs) in which an endogenous amino acid coordinates the ligand binding site of the heme iron in the absence of exogenous ligands. The fact that hxHbs are capable of reversible exogenous ligand binding distinguishes them from cytochrome b₃ and denatured Hbs that are not capable of ligand binding in the hexacoordinate state (12, 14–16). This unusual characteristic could be an alternative form of regulating ligand affinity (17, 18) or indicate a different functional role for hxHbs compared with the traditional role of oxygen storage and transport (19–22). Although hxHbs are found in many species, few structures have been reported in the hexacoordinate state. Thus, further investigation of structures across a wide variety of species will aid in understanding reversible hexacoordination and discovering its physiological significance.

The hemoglobin found in the single-celled cyanobacterium Synechocystis sp. PCC 6803 (SynHb) belongs to the truncated hemoglobin (trHb) family found extensively in eubacteria, bacteria, single celled eukaryotes, and plants (2, 23–26). The trHbs are 20–40 residues shorter than non-vertebrate hemoglobins and have a 2-on-2 a-helical globin fold rather than the typical 3-on-3 fold. They are further characterized by a hydrophobic tunnel connecting the protein surface to the distal heme pocket that could serve as a direct route for ligand entry and/or exit from the heme pocket (27). In addition to being a hexacoordinate member of the trHb family, SynHb is distinguished by the ability to form a covalent bond between the heme 2-vinyl group and the His¹¹⁷ side chain (27); this is a novel example of covalent attachment in a Hb via the porphin ring, and the purpose of this modification is not yet understood.

In the present study, the crystal structure of SynHb has been solved to 1.8-Å resolution. The crystal structure reveals the covalent linkage between the heme 2-vinyl and the Nε2 atom of His¹¹⁷ that is not present in the solution NMR structure of this protein (28). The crystal structure is compared with the solution NMR structure and to other trHb and hxHb structures in an effort to understand the role of this unusual covalent mod-

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The atomic coordinates and structure factors (code IRTX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: Hb, hemoglobin; Mb, myoglobin; SynHb, Synechocystis sp. hemoglobin; haHb, hexacoordinate hemoglobin; trHb, truncated hemoglobin; r.m.s.d., root mean square deviation; CtrHb, C. elegans truncated hemoglobin; PrtHb, P. caudatum truncated hemoglobin; trHBbN, M. tuberculosis truncated hemoglobin; RiceHb, rice hemoglobin 1; Ngb, neuroglobin; Hb-C, hemichrome chain from C. arcenica; eHb, hemichrome chain from horse methemoglobin; HbTn, hemichrome chain from T. neuvesis; ICYT, bovine cytochrome b₃; rCYT, rat cytochrome b₃; HSO, cytochrome b₃ domain from human sulfite oxidase; CSO, cytochrome b₃ domain from chicken liver sulfite oxidase; MES, 4-morpholinoethanesulfonic acid; Lba, leghemoglobin.
tein was oxidized with a slight molar excess of potassium ferricyanide conditions similar to those in which the His 117-heme covalent link was observed previously (18, 24) with the following exceptions. Expression was performed without induction by isopropyl-1-thio-β-D-galactopyranoside, and free hemin was not added during fermentation. Following inoculation, cells were grown for 16 h and then harvested by centrifugation. The resulting supernatant was bright red due to soluble heme-bound SynHb existing predominately in the ferrous state. Protein purification proceeded as described previously (18), resulting in protein with a Soret/A280 absorbance ratio greater than 5.0. The purified protein was oxidized with a slight molar excess of potassium ferricyanide followed by desalting on a Sephadex G-25 column in 0.1 M potassium phosphate, pH 7. The resulting ferric SynHb was concentrated to ~3 mM and stored at ~80 °C until use.

The ferric protein used for crystallization was produced under conditions similar to those in which the His117-heme covalent link was observed by NMR experiments in SynHb and the homologue in Synchococcus (28, 29). However, the reported method of dithionite treatment leading to the covalent link (27, 28) was not used in our treatment of the protein. Crystal growth was achieved by hanging-drop vapor diffusion. Drops were produced by mixing 2 μl of 3 mM protein with 2 μl of well buffer containing 30–35% polyethylene glycol monomethylether 5000, 0.2 M ammonium sulfate, 0.01 M cadmium chloride, and 0.1 M MES at pH 6.5. Single crystals grew in 1–3 days at 4 °C.

**Structure Determination and Refinement**—Initially, solution of the crystal structure of SynHb was attempted using molecular replacement starting with the NMR structure of this protein (28). The fact that this method failed is not unusual because the use of NMR structures for starting with the NMR structure of this protein (28). The fact that this crystal structure of SynHb was attempted using molecular replacement starting models is often unsuccessful, even with the NMR structure of SynHb in that the B and E, and G and H helices form the characteristic 2-on-2 helical core of the globin fold (2) (Fig. 1A). The covalent linkage between the Nε2 atom of His117 and the heme-2-vinyl is observed as continuous electron density between these atoms (Fig. 1B). There is no evidence for a mixture of states for this side chain, suggesting that the protein is exclusively in the covalently cross-linked conformation. The Nε2 atom of His117 is 2.1 Å from the CAB atom of the heme in the crystal structure, whereas these atoms are 5.4 Å apart in the most representative model from NMR. This movement is accompanied by a 136° change in the angle of CHI2 and a 136° change in the angle of CHI3.

**RESULTS**

**The Crystal Structure of SynHb in Comparison to the Solution NMR Structure**—The backbone structure and electron density for SynHb are shown in Fig. 1. The overall structure is typical of other trHbs and the NMR structure of SynHb in that the B and E, and G and H helices form the characteristic 2-on-2 helical core of the globin fold (2) (Fig. 1A). The covalent linkage between the Nε2 atom of His117 and the heme-2-vinyl is observed as continuous electron density between these atoms (Fig. 1B). There is no evidence for a mixture of states for this side chain, suggesting that the protein is exclusively in the covalently cross-linked conformation. The Nε2 atom of His117 is 2.1 Å from the CAB atom of the heme in the crystal structure, whereas these atoms are 5.4 Å apart in the most representative model from NMR. This movement is accompanied by a 136° change in the angle of CHI2 and a 136° change in the angle of CHI3.

Another distinct feature of the SynHb crystal structure is the relatively large r.m.s.d. from the NMR structure, as illustrated in Figs. 2 and 3. Two methods were used to align the structures for quantitative comparison. The first method aligns the structures based on the Ca atoms of 109 residues (89% of the residues in the SynHb structure) with clear backbone density in the crystal structure (residues 5–55, 60–98, 105, and 107–124). The second method aligns the structures based on the Ca atoms of 86 residues (70%) involved in clear secondary structure (residues 17–55, 64–98, and 101–112) and was chosen...
because it was the method used for aligning the 20 NMR models of SynHb (28).

The r.m.s.d. values for the density-based and secondary structure-based alignments were relatively high at 2.18 and 1.6 Å, respectively. The difference in r.m.s.d. values between the two methods arises from the number of residues used for each alignment. Both alignments give an r.m.s.d. of 2.6 Å when all 123 Cα atoms of the identical sequence are taken into account, rather than only the subset of Cα atoms used in the alignment. As a frame of reference, the average r.m.s.d. among the 20 NMR models relative to the most representative structure is 1.37 Å for all Cα atoms. In addition, the comparison between the crystal and NMR structures of SynHb gives a higher r.m.s.d. value than the comparison of the SynHb crystal structure and that of the trHb from Chlamydomonas eugametos (CtrHb). The latter comparison aligns 94 Cα (76%) with an r.m.s.d. of 1.53 Å, even though CtrHb is in a different liganded state than SynHb and does not share the same primary sequence.

The source of deviation between the crystal and NMR structures is examined on a per residue basis in Fig. 2. Fig. 2A includes a plot of the r.m.s.d. between the SynHb crystal and NMR structures. Fig. 2B is a ribbon representation of the crystal structure backbone onto which r.m.s.d. values have been color-coded for each amino acid (blue is <1.5 Å, orange 1.5–3 Å, and red >3 Å). Several regions of increased r.m.s.d. are expected because of increased mobility. These regions include the termini and extended loops, both of which correlate with increased B factors in Fig. 2A. There are also regions described by relatively low B factors that have increased r.m.s.d., including the entire F helix, the first half of the E helix, and a portion of the lower H helix containing the covalently linked His117. These differences between the crystal and NMR structures of SynHb are seen clearly in the backbone alignment shown in Fig. 3A.

In the case of hemoglobins, it can be argued that structural alignment based on the heme prosthetic group is the most important functionally, because it gives a direct comparison of the orientation of the amino acid side chains influencing the steric and electrostatic environment immediately surrounding the ligand binding site. Alignment based on heme position rather than the protein backbone increases the deviation between the crystal and NMR structures of SynHb from 2.6 Å to 3.76 Å for all Cα. This indicates that the heme orientations within the crystal and NMR structures of SynHb are different (as can also be seen in Fig. 3A). Both the Cα and heme-based alignment comparisons indicate significant deviations between the crystal and NMR structures of SynHb, particularly at high resolution.

Inclusion of side chain positions in the backbone comparison of these structures also generates r.m.s.d. values > 3 Å. Differences occur throughout the backbone but are most pronounced in the heme pocket overlay shown in Fig. 3B, which provides a direct comparison of the crystal structure and the most representative NMR structure. Several side chains, including Tyr22 and Phe35, are shifted upwards, away from the heme in the NMR structure. The H helix is closer to the heme in the crystal structure, which brings His117 into position to form a covalent bond to the heme 2-vinyl group. Additional high resolution structural differences include the position of the side chains of the proximal and distal histidines, His70 and His117, respectively (Table II), which have different orientations with respect to the heme porphyrins in the two structures. The proximal histidine in the crystal structure is tilted toward the propionates with an acute angle of 79° between the least squares planes of the heme porphyrin and the histidine imid-
azole ring, whereas in the NMR structure the proximal histidine is tilted away from the propionates with an acute angle of 77°. The effect of this tilt on the azimuthal angle of the proximal histidine is discussed below. Similarly, the tilts of the distal histidines are nearly opposite; that in the crystal structure tilts toward the 1- and 8-methyl groups with an acute angle of 73°, whereas the one in the NMR structure tilts toward the 4-vinyl and 5-methyl groups with an acute angle of 76°.

**Solvent Accessibility in SynHb**—The crystal structures of three other group I trHbs have been solved to date, CtrHb, *Paramecium caudatum* (PtrHb), and *Mycobacterium tuberculosis* (trHbN) (13, 43), all with bound ligands. Each of these structures display a continuous or nearly continuous “ligand tunnel” that connects the distal heme pocket with solvent. Utilizing the same parameters for tunnel calculations in the other trHbs (see “Experimental Procedures”), SynHb does not contain this tunnel. In the SynHb crystal structure, crowding from residues such as Leu51, Phe55, and Met98 narrows the space between the AB and GH inter-helical corners and the distal heme pocket, whereas Phe50 blocks access to the distal pocket via this path completely. Furthermore, Tyr53 in SynHb prevents access to the heme from the branch of the tunnel found between the G and H helices. For the NMR structure, it was reported that this tunnel path was detected (28). However, when the same method was again used to check for solvent accessibility in the NMR structure, it was found that, despite the porous surface of the protein, no continuous tunnels extend through the protein matrix to the distal heme pocket.

Both the crystal and NMR structures of SynHb are solvent-accessible around the heme propionates. The solvent-accessible cavity of the crystal structure (Fig. 4A) is smaller than that of the NMR structure (Fig. 4B), which is more extensive and reaches into the heme pocket to surround the distal His46.

### Table II

| Protein                | B10  | CD1  | E7   | E10 | E11 | E14 | F8   |
|------------------------|------|------|------|-----|-----|-----|------|
| *Synechocystis*        | Tyr22| Phe35| Gln43| His46| Gln47| Phe50| His70|
| *C. eugametos*        | Tyr20| Phe33| Gln41| Lys44| Gln45| Phe55| His56|
| *P. caudatum*         | Tyr20| Phe33| Gln41| Lys44| Thr45| Phe55| His55|
| *M. tuberculosis*     | Tyr33| Phe50| Leu54| Gln56| Phe56| His51|   |

**Fig. 3.** The crystal structure of SynHb compared with the NMR structure. A, a cross-eyed stereo overlay of the crystal structure of SynHb in red and the most representative NMR structure in blue, with helices labeled; alignment is based on the Cα atoms of 109 residues with clear backbone density. B, a crossed-eyed stereo overlay of the heme pocket, aligned with respect to the heme group.
However, the rest of the heme group is blocked from solvent as is the case in other trHbs. For example, in the crystal structures of CtrHb and PtrHb, the side chains of Phe^{48} and Trp^{59} block the heme from solvent accessibility at the CHD methinic bridge (13). In the crystal structure of SynHb, Tyr^{53} and Tyr^{61} guard this side of the heme from solvent. In the NMR structure of SynHb, Tyr^{53} also serves this function but Tyr^{61} does not due to the wide swing of the pre-F loop in this conformation (28).

**Comparison of SynHb to the Structures of Other trHbs**—SynHb is the first crystal structure of a trHb in the unliganded state. Of the three crystal structures previously solved for other group I trHbs, SynHb shares highest sequence identity with CtrHb (34%) and somewhat lower sequence identities with trHbN (24%) and PtrHb (20%) (25). The overlay of SynHb and CtrHb is shown in Fig. 5A. SynHb shares a 2-on-2 α-helical fold more similar to that of CtrHb and PtrHb than trHbN (respective r.m.s.d. values are 1.53 Å over 94 Ca, 1.65 Å over 85 Ca, and 2.16 Å over 51 Ca). All four of these trHbs exhibit the characteristic three glycine motifs, the shortened one-turn A helix (though trHbN also has a N-terminal extension), and significant deletions in the CD-D region.

General differences between the SynHb crystal structure and other trHbs include a shift of the SynHb B helix away from the heme group, and movement of the E helix to a position closer to the heme that facilitates the bond between His^{46} and the distal site of the heme iron. The F helices in other trHb crystal structures contain a wide “pre-F” loop preceding a one-turn F helix hosting the proximal His (F8) (“F8” designates the helix position of the proximal His; see Table II). In contrast, the pre-F loop in SynHb is shorter and closer to the heme due to more extensive helical structure in this region. The F helix in the crystal structure of SynHb begins with one and a half turns (residues F1–F6 as opposed to residues F4–F8 in the others), is interrupted by a three residue loop hosting the His (F8), and ends in a one-turn loop of four residues. This is different from the NMR structure of SynHb, in which the pre-F loop swings out in the opposite direction from the three ligand bound structures, and the F helix is one long, 13-residue helix beginning at F2. Finally, the H helix in SynHb is interrupted and bent by a loop (similar to that in PtrHb) that brings the end of the H helix close to the heme at the His^{17} covalent linkage.

Differences in the locations of specific, functionally important heme pocket amino acids in the crystal structure of SynHb compared with those of the other trHb proteins can be summarized as follows. 1) His(F8), Phe(CD1), and Tyr(B10) are conserved in all four structures (Table II). The Tyr(B10) side chain is found in the distal heme pocket and acts to stabilize the ligand in the other trHbs (13, 43–45) but extrudes out into the solvent in the SynHb structure. 2) Gln(E7) in CtrHb and PtrHb also stabilizes ligand binding (13), but in SynHb, Gln(E7) extrudes out into the solvent, up and away from the heme, and forms hydrogen bonds with two solvent molecules. 3) Thr(E11) in PtrHb and Gln(E11) in both CtrHb and trHbN participate in a network of hydrogen bonds that includes the side-chain hydroxyl of Tyr(B10) in each protein (13, 43). Gln(E11) in SynHb is located well above the heme and hydrogen bonds to a solvent molecule. 4) In the other trHb structures, the Lys(E10) side chain extrudes out into the solvent, interacting with the heme propionates in CtrHb and PtrHb (13). The corresponding His(E10) side chain in SynHb binds the distal site of the heme iron. In summary, besides hexacoordination, the major difference among the heme pockets of these structures is that several side chains important for stabilizing the bound ligand in other trHbs are found interacting with solvent in SynHb.

**Comparison of SynHb with Other Hexacoordinate Hemoglobins**—The structures of hemoglobins in the hemichrome state from five other organisms have been reported. These are the Hb-C chain from the sea cucumber *Caudina arenicola* (Hb-C) (46), rice non-symbiotic Hb (RiceHb) (12), a tetrameric hemoglobin from the Antarctic fish *Trematomus newnesi* (HbTn) (47)
(with the β chains in a hemichrome state), horse methemoglobin (eHb) at pH 5.4 (with the α chains in a hemichrome state) (48), and human neuroglobin (Ngb) with three Cys mutations (11). Backbone overlays of these hxHbs (none of which are trHbs) do not align well with SynHb. However, SynHb looks most like a truncated version of RiceHb, with an r.m.s.d. of 1.71 Å over 89 Cα atoms (Fig. 5B).

In the other hxHbs the hexacoordinating side chain is the distal His(E7), but in the two structures of SynHb the hexacoordinating distal residue is His(E10). Heme pocket comparisons reveal further interesting differences in stereochemistry among these hxHbs (Fig. 6 and Table III). For example, Fig. 6 shows the azimuthal angles for the above hxHbs and the two SynHb structures. Myoglobin (Mb) (49) and leghemoglobin (Lba) (50) are also shown, along with four cytochrome b5 proteins; bovine (bCYT) and rat (rCYT) cytochrome b5 (51, 52), and the cytochrome b5 domains from human (HSO) and chicken liver (CSO) sulfite oxidases (53, 54).

The azimuthal angle is defined as the intersection of the least squares plane of the proximal histidine with the least squares heme plane, relative to a line connecting diagonally opposed pyrrole nitrogens. The planar intersections are presented as colored lines through a representative heme molecule in Fig. 6A. The intersection for Mb (light cyan) eclipses the pyrrole nitrogens, whereas Hb-C (dark cyan) nearly eclipses the opposite pyrrole nitrogens, and Lba (orange), RiceHb (purple), and Ngb (yellow), are staggered to various degrees. However, the azimuthal angles for eHb (pink), HbTn (gray), the crystal structure of SynHb (red), and the NMR structure (blue) are staggered but do not pass through the iron atom, as shown in Fig. 6B. This is due to the tilt of the proximal histidine imidazole plane. The tilts for all of the proteins in Fig. 6A, as delineated in the first column of Table III, are within about 2° of perpendicular to the heme plane. However, the proteins in Fig. 6B have tilts of 7° to 14° from the normal to the heme
The conventional "histidine-gate" path for ligand binding is blocked in trHbs (2). It has been proposed that the tunnel found in the previous trHb structures serves as an alternative diffusion path for ligands (43). However, there is no protein matrix tunnel in SynHb. Therefore, three possibilities exist for ligand entry and exit in SynHb. 1) Ligands enter and exit through the solvent face of the heme pocket. 2) Ligands enter through the solvent face of the heme pocket, and then a tunnel forms in the ligand bound state that serves as a route for exit. 3) Ligand entry and exit pathways are not evident in these structures. The solvent accessibility around the propionates would be an appropriate route for ligand access in support of the first two possibilities. In the second case, formation of a tunnel after ligand binding followed by its blockage when the ligand dissociates could serve to trap ligands near the heme pocket, providing a barrier to ligand escape, and enhancing germinate ligand recombination. This possibility is supported by the observation of room temperature CO germinate recombination in SynHb (24, 45).

**Implications for Ligand Binding in trHbs—SynHb is similar in overall fold to other trHbs with the obvious exception of intramolecular heme iron coordination. This is likely to account for many structural differences with other trHbs, such as the downward shift of the B helix that moves Tyr(B10) into position to stabilize the ligand in other trHbs. Because Tyr(B10) in SynHb is also predicted to stabilize bound ligands (24), a similar downward shift of the B helix is expected upon ligand binding in SynHb. His46 is predicted to be uninvolved in ligand stabilization (24, 25). This suggests that ligand binding in SynHb will cause a shift in the position of the E helix that moves this side chain outwards into the solvent. This shift could also move the Glu(E7) and Glu(E11) residues into position to contribute to a hydrogen bonding network around the bound ligand and Ala residues of the ligand bound structures of other trHbs (2).

Although these structural changes would cause ligand-bound SynHb to resemble other ligand-bound trHbs, the ligand-free structures of CtrHb, PtrHb, and trHbN must be different than that of SynHb. Of these three, only CtrHb is capable of hexacoordination, which occurs only at alkaline pH by the Tyr(B10) side chain (55). This suggests that the B helix rather than the E helix in CtrHb moves closer to the heme in the absence of exogenous ligands. Structures of PtrHb and trHbN in the pentacoordinate state would be different from both SynHb and CtrHb in that neither exhibits hexacoordination.

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ligands. Hexacoordination in SynHb results from a different tertiary arrangement of the B and E helices compared with other hxHbs, as well as many specific differences in heme pocket stereochemistry, leading to alternative mechanisms for regulating ligand binding. Examination of SynHb in the context of other hxHbs and cytochrome b$_6$ reveals that destabilization of the distal His-heme iron bond to allow for exogenous assistance.

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REFERENCES

1. Gardner, P. R., Gardner, A. M., Martin, L. A., and Salzman, A. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10378–10383
2. Wittenberg, J. B., Wittenberg, B. A., and Guertin, M. (2002) J. Biol. Chem. 277, 871–874
3. Kunda, S., Trent, J. T., III, and Hargrove, M. S. (2003) Trends Plant Sci. 8, 387–393
4. Burmester, T., Welch, B., Reinhardt, S., and Hankeln, T. (2000) Nature 407, 520–523
5. Trent, J. T., III, and Hargrove, M. S. (2002) J. Biol. Chem. 277, 19538–19545
6. Trent, J. T., III, Watts, R. A., and Hargrove, M. S. (2001) J. Biol. Chem. 276, 30106–30110
7. Burmester, T., Eberh, B., Welch, B., and Hankeln, T. (2002) Mol. Biol. Ecol. 19, 416–421
8. Minning, D., Gow, A., Bonaventura, J., Braun, R., Dohr, M., Heng, D., and Stammer, J. (1999) Nature 401, 497–502
9. Hou, S., Larsen, R., Bouloudikou, D., Riley, C., Zimmer, K., Ortega, G., and Alam, M. (2000) Nature 404, 540–544
10. Burr, A., Hunt, P., Wagar, D., Dewide, S., Blaxter, M., Vafier, J., and Moens, L. (2000) J. Biol. Chem. 275, 4810–4815
11. Pesce, A., Dewide, S., Nardini, M., Moens, L., Aschenz, P., Hankeln, T., Burmester, T., and Bolognesi, M. (2003) Structure (Camb) 11, 1087–1095
12. Hargrove, M., Brucker, E., Stee, B., Sarath, G., Arredondo-Peter, R., Kukas, E., Olson, J., and Philipp, G. (2006) Structure Fold. Des. 8, 1005–1014
13. Pesce, A., Couture, M., Dewide, S., Guertin, M., Yamauchi, K., Aschenz, P., Moens, L., and Bolognesi, M. (2000) EMBO J. 19, 2424–2434
14. Rixon, J. M., Abou, O., Levy, A., and Heim, J. (1994) Methods Enzymol. 231, 449–488
15. Antonini, E., and Brunori, M. (1971) Hemoglobin and Myoglobin in Their Reactions with Ligands, North-Holland Publishing Company, Amsterdam
16. Dou, Y., Aden, S. J., Ikeda-Saito, M., Kryzewa, S., Wilkinson, A. J., Li, T., Olson, J. S., Prince, R. C., Pickering, J. I., and George, G. N. (1995) J. Biol. Chem. 270, 15993–16001
17. Hargrove, M. S. (2000) Biochimie 79, 2373–2378
18. Trent, J. T., III, Hvitved, A., and Hargrove, M. S. (2001) Biochemistry 40, 6155–6160
19. Duf, S. M., Wittenberg, J. B., and Hill, R. D. (1997) J. Biol. Chem. 272, 16746–16752
20. Arredondo-Peter, R., Hargrove, M. S., Sarath, G., Moran, J. F., Lohrmann, J., Olson, J. S., and Kukas, R. V. (1997) Plant Physiol. 115, 1239–1246
21. Tretiakov, B., Watts, R. A., Anderson, C. R., Llewellyn, D. J., Hargrove, M. S., Olson, J. S., Dennis, E. S., and Peacock, W. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12320–12324
22. Kunda, S., Premer, S., Hoy, J., Trent, J. T., and Hargrove, M. (2003) Biophys. J. 84, 3931–3940
23. Scott, N., and LeComte, J. (2000) Protein Sci. 3, 587–597
24. Hvitved, A. N., Trent, J. T., III, Premer, S. A., and Hargrove, M. S. (2001) J. Biol. Chem. 276, 34714–34721

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