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Chemical reactivity of *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 hemoglobins: covalent heme attachment and bishistidine coordination

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Abstract

In the absence of an exogenous ligand, the hemoglobins from the cyanobacteria *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 coordinate the heme group with two axial histidines (His46 and His70). These globins also form a covalent linkage between the heme 2-vinyl substituent and His117. The in vitro mechanism of heme attachment to His117 was examined with a combination of site-directed mutagenesis, NMR spectroscopy, and optical spectroscopy. The results supported an electrophilic addition with vinyl protonation being the rate-determining step. Replacement of His117 with a cysteine demonstrated that the reaction could occur with an alternative nucleophile. His46 (distal histidine) was implicated in the specificity of the reaction for the 2-vinyl group as well as protection of the protein from oxidative damage caused by exposure to exogenous H₂O₂.

Keywords

Truncated hemoglobin; Heme posttranslational modification; Hybrid *b/c* heme; Reactive oxygen species; Cytochrome *c*

Introduction

Hemoglobins are small helical proteins containing one *b* heme group per polypeptide chain. The textbook hemoglobin subunit harbors a ferrous iron coordinated by four pyrrole nitrogens from the porphyrin ring and the Nε2 atom of the proximal histidine. In the oxy state, dioxygen completes the iron octahedral geometry, whereas in the deoxy state, the distal site either remains vacant or is occupied by a loosely bound water molecule [1]. The hemoglobin heme pocket is such that reversible binding of dioxygen occurs with low probability of side reactions, such as iron oxidation, that would impair function. Not all hemoglobins, however, exhibit these structural and chemical properties. A growing number of characterized members of the superfamily are now known to use a protein side chain as an axial ligand to the ferrous iron on the distal side when the partial pressure of dioxygen is low [2–6]. It has also become clear that many bacterial and plant globins associate with dioxygen and other small molecules for enzymatic purposes rather than delivery [7].

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Variations in heme structure, environment, and distal iron ligation are all exemplified in the 2/2 (truncated) lineage of the hemoglobin superfamily, a set of proteins for which there is thus far little functional information, but which holds promises of novel heme chemistry [7].

The 2/2 hemoglobins of the cyanobacteria *Synechococcus* sp. PCC 7002 (*Synechococcus* 7002 hereafter) and *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) contain 123 amino acids and differ at 50 positions scattered throughout the sequence. Physiological studies of *Synechococcus* 7002 show that its hemoglobin (called GlnN) participates in protection against reactive oxygen species (ROS) or reactive nitrogen species (RNS), likely peroxynitrite or nitric oxide or both [8]. Similar in vivo information is not available for *Synechocystis* 6803 GlnN, but a strain of *Escherichia coli* lacking the essential native flavohemoglobin gene (*hmp*) and complemented with the *glnN* gene is viable. Thus, it appears that *Synechocystis* 6803 GlnN can take on the role of the heme domain of flavohemoglobin in converting NO into nitrate [9].

Both GlnNs exhibit bishistidine coordination of the heme iron in the ferrous and ferric resting states [10, 11]. Their fold is otherwise typical of 2/2 hemoglobins belonging to phylogenetic group I [12]. Surprisingly, the two cyanobacterial GlnNs also undergo a chemical modification by which the heme is irreversibly attached to the protein. The cross-link involves the C α of the vinyl group on pyrrole B and the N ϵ 2 atom of His117 [13, 14] as depicted in Fig. 1a. Wild-type recombinant GlnNs (rGlnNs) prepared by reconstitution of the apoprotein with iron(III) protoporphyrin IX (rGlnN-R hereafter) are efficiently converted to the adduct (rGlnN with covalently attached heme, rGlnN-A) by addition of the reducing agent dithionite (DT) [14]. The cross-link appears to have physiological significance as *Synechococcus* 7002 cells contain higher levels of GlnN-A when grown under micro-oxic conditions than when grown under standard oxic conditions [8].

Although posttranslational heme vinyl modifications have not been observed in any hemoglobin other than the GlnNs discussed here, heme vinyl groups are moderately reactive and are natural sites for modification in other proteins. The most familiar example is provided by c-type cytochromes, which are biosynthesized from apocytochrome and b heme and contain one or two thioether bonds to cysteines. Interestingly, the maturation of cytochrome c via system I (CcmABCDEFGH) proceeds with transient heme attachment to a histidine or a cysteine of CcmE [15]. The nature of the CcmE histidine adduct has been elucidated: the reversible linkage is between the N δ 1 atom and the heme 2-C β [16], unlike in GlnN-A. Nonnative attachment of a protein residue at a heme vinyl has also been proposed in the His25SeCys variant of human heme oxygenase 1 (C β position [17]), the Ser160Met variant of ascorbate peroxidase under oxidative conditions (C β position [18]), and the Ser160Cys variant of the same under reducing conditions [19]. In GlnN, radical and nonradical as well as reductive and oxidative routes are all a priori plausible.

Synechococcus 7002 and *Synechocystis* 6803 GlnNs offer an opportunity to examine the chemistry associated with covalent heme attachment and bishistidyl coordination. These proteins are particularly well suited for analysis because they can be prepared, and are sufficiently stable, in the apoprotein state and the holoprotein state with or without heme covalent attachment. Here, we investigate the reactivity of rGlnNs toward H₂O₂, an ROS generated during aerobic metabolism in cyanobacterial cells, and propose a mechanism for the reduction-driven formation of the heme–protein cross-link. The new data expand our view of the chemistry supported by the globin fold.

Materials and methods

The procedures for production and purification of recombinant cross-linked and non-cross-linked Glns (rGln-R and rGln-A, respectively) have been published previously [11, 20] and were followed with minor changes. Hemochromogen assays [21, 22] and acid–butanone heme extractions [23] were performed as reported in prior work. Details are provided in the electronic supplementary material.

Electronic absorption spectroscopy

Absorbance data were collected using one of three instruments, an AVIV Instruments model 14-DS spectrophotometer equipped with a Peltier device for temperature control, an Olis RSM-1000 UV–vis spectrometer equipped with a stopped-flow rapid mixing apparatus, or a Varian Cary 50 spectrophotometer. All samples were prepared in degassed buffer, and ranged in concentration from 10 to 70 μM .

rGln reduction under micro-oxic conditions

When sensitivity to dioxygen was a concern, the ferrous state was generated in the presence of the glucose oxidase/D-glucose/catalase (GODCAT) system [24]. Glucose oxidase (*Aspergillus niger*), catalase (bovine liver) and D-(+)-glucose were purchased from Sigma. Typical solutions used for UV–vis experiments included 300 nM glucose oxidase, 70 nM catalase, and 30 mM D-(+)-glucose in 100 mM sodium phosphate, pH 7.2. The solutions were incubated for 1.5 h before the addition of a concentrated protein solution followed by a second 1-h-long incubation period prior to reductant addition. The GODCAT system removes dissolved dioxygen to steady-state concentrations in the 10–20 μM range [25]. Catalase is sensitive to dithiothreitol (DTT) [26], but control experiments did not indicate this to be a concern under our conditions.

H₂O₂ treatment

H₂O₂ (30% Fischer) was diluted with doubled-distilled H₂O to produce 1–100 mM stock solutions. The concentration of H₂O₂ was determined using the extinction coefficient $\epsilon_{240\text{ nm}} = 39.4\text{ M}^{-1}\text{ cm}^{-1}$ [27]. Protein solutions contained approximately 100 mM phosphate, pH ~ 7.2. For UV–vis spectrophotometry experiments, protein sample concentrations were 5–10 μM . A fivefold excess of H₂O₂ was added to the protein solution in a stoppered cuvette. After 10–15 s of manual mixing, absorption spectra were collected using a Varian Cary 50 from 430 to 401 nm, with a 0.1-s averaging time, at room temperature, as a function of time. For cyanide protection assays, cyanomet rGlns were prepared by incubating proteins with a fivefold to 500-fold excess of KCN prior to treatment with H₂O₂.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis was performed with approximately 50 μM protein samples. For kinetic experiments, protein samples were treated with 5 equiv of H₂O₂ and aliquots were extracted as a function of time. At the end of each incubation period, reactions were quenched by addition of sodium dodecyl sulfate sample buffer supplemented with 360 mM β -mercaptoethanol (final concentration) and boiled for 5 min prior to electrophoresis. In the second type of experiment, samples were exposed to 0.5–100 equiv of H₂O₂ and incubated for 3 h at room temperature prior to quenching, denaturation, and electrophoresis.

Fluorescence emission

A fivefold to 100-fold excess of H₂O₂ was added to an approximately 100 μM protein solution and the mixture was incubated for 3 h at room temperature. Following incubation, the samples were subjected to an acid–butanone heme extraction. The recovered apoprotein

was dialyzed extensively against H₂O to remove any 2-butanone. The protein was diluted to approximately 2 μ M in 100 mM phosphate, pH 7.2. Fluorescence data were acquired with an Aviv ATF 105 automated titrating differential/ratio spectrofluorometer at 25 °C with excitation at 315 nm. The fluorescence emission spectrum was recorded from 325 to 500 nm in 1-nm steps with a 2-s averaging time.

Formation of the heme–protein cross-link, manual mixing

For manual mixing experiments with DTT and *Synechococcus* 7002 rGlbN, the solid reagent was dissolved in a buffer preincubated with GODCAT, as described earlier, under an N₂ atmosphere and so as to reach a 500 times stock. Immediately after the DTT had solubilized, 2 μ L was added to a 998- μ L solution containing GODCAT-incubated protein in a quartz cuvette for absorbance measurement. The cuvette was then stoppered, inverted several times, and sealed with Parafilm. The cuvette was removed from the nitrogen atmosphere and placed in a Varian Cary 50 spectrophotometer for data collection. Dead times ranged between 30 and 50 s. The spectra were recorded in the range 700–260 nm in 1-nm steps, with a 0.2-s averaging time, and over a minimum of 24 h.

Manual mixing experiments were also performed with DT and *Synechocystis* 6803 rGlbN-R in the presence and absence of the GODCAT scavenger. Solid DT was dissolved in the appropriate buffer to produce a 1 M stock and was added to yield a fourfold to 200-fold excess of DT over protein. DT concentrations were estimated by mass and verified by optical spectroscopy using the extinction coefficient $\epsilon_{314\text{ nm}} = 8\text{ mM}^{-1}\text{ cm}^{-1}$ [28]. The dead time was 10–30 s. The spectra were recorded over the range 500–600 nm with 1-nm steps. Spectra were collected every 20 s. The total acquisition time ranged from 20 min to 2 h.

Formation of the heme–protein cross-link, stopped-flow rapid mixing

To eliminate the possibility that dioxygen was involved in the reaction, kinetic data were collected by stopped-flow rapid mixing with an Olis RSM-1000 UV–vis spectrometer in an anaerobic chamber. The proteins were wild-type and H117A *Synechocystis* 6803 rGlbN. Dioxygen was removed from 100 mM tris(hydroxymethyl)aminomethane pH 8.2 through a combination of N₂ sparging and vacuum exposure. Stocks of approximately 70 μ M rGlbN-R were freshly prepared from lyophilized proteins. The initial concentrations were measured with injections of protein stock solutions and buffer containing no DT ($\epsilon_{545\text{ nm}} = 10.67\text{ mM}^{-1}\text{ cm}^{-1}$). An approximately 90 μ M DT solution was prepared by dissolving solid DT in deoxygenated buffer. The stock solution was prepared in the anaerobic chamber and transferred to a cuvette with a sealable Teflon valve that was closed before the cuvette was removed from the chamber. The stock solutions were quantitated with a Hewlett-Packard photodiode-array spectrophotometer outside the anaerobic chamber.

Equal volumes of DT and protein were subjected to rapid stopped-flow mixing. Scans were collected every 0.1 s for 3 min; the wavelength range was 438–662 nm, covered in 1-nm steps. The raw data were subjected to singular value decomposition following the method of Henry and Hofrichter [29] using the program Scilab 5.2.2 (<http://www.scilab.org/>). The abstract kinetic vectors were globally fit with the program Savuka [30].

NMR experiments

NMR samples were prepared in 20 mM phosphate buffer, pH 7.2, at 25 °C (90:10 H₂O/D₂O, or 99.9% D₂O) unless specified otherwise. Protein concentrations were 0.1–5 mM. Samples of CO-bound rGlbN were prepared by saturating the protein solution with CO, then adding a buffered DT solution also saturated with CO. This procedure resulted in samples containing a mixture of rGlbN-R and rGlbN-A.

NMR spectra were collected with Bruker DRX-600 and Avance 600 spectrometers and a Varian Inova 500 spectrometer. ^1H chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid through the water resonance with correction for temperature. Double-quantum-filtered correlation spectroscopy, nuclear Overhauser effect spectroscopy, water-elimination Fourier transform nuclear Overhauser effect spectroscopy, total correlation spectroscopy, ^1H - ^{13}C heteronuclear multiple quantum coherence (HMQC), and ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) data were collected as described previously [11, 14, 20].

To establish the nature of the covalent attachment in H46L *Synechococcus* 7002 rGln-A (see “Results”), long-range selective ^1H - ^{15}N HMQC data were collected on uniformly ^{15}N labeled ferric protein in the cyanomet state (1 mM protein, pH 7.1 or 6.4, 25 °C) using a 22-ms magnetization transfer delay. Selective 1-ms ^{15}N $\pi/2$ pulses were utilized to minimize backbone excitation. To determine the structure of the vinyl modification in H117C *Synechocystis* 6803 rGln-A, natural abundance ^1H - ^{13}C non- $\{^1\text{H}\}$ -decoupled HSQC data were collected on the ferric protein (3 mM protein, pH* 8.7, 25 °C in 99.9% D_2O) using a 1.3-ms transfer time. A 2-ms selective ^{13}C refocusing π pulse was used to achieve band selection in the indirect dimension. ^1H - ^{13}C coupling was established by omitting the ^1H π pulse centered in t_1 . Data of the same kind were collected to identify the CH_2D group in the wild-type protein allowed to react in D_2O . In this case, a sensitivity-enhanced version of the pulse sequence was utilized.

NMR data were processed using XWIN-NMR, TOPSPIN (Bruker BioSpin, Rheinstetten, Germany), and NMRPipe [31]; spectra were analyzed with TOPSPIN and Sparky [32].

Results

The NMR structure of *Synechococcus* 7002 rGln-A is shown in Fig. 1b [8]. The 50 amino acid substitutions distinguishing *Synechococcus* 7002 Gln from *Synechocystis* 6803 Gln do not change the fold and are responsible for modest variations in thermodynamic stabilities, ligand binding properties, and chemical reactivity. Accordingly, we found that one protein occasionally gave clearer results than the other in selected experiments. However, several conclusions do not depend on the protein choice. These are emphasized throughout.

The role of His46 in the posttranslational modification

The sole detectable product of the heme modification caused by DT reduction of ferric wild-type *Synechocystis* 6803 rGln-R was identified in prior NMR work as the Markovnikov adduct (*R* stereochemistry) of His117 onto the heme 2-vinyl [13, 14] (Fig. 1a). When the starting material is ferric H46L rGln-R, H46A rGln-R, or the cyanomet complex of wild-type rGln-R, DT reduction produces the 2-vinyl adduct and a measurable amount of the 4-vinyl adduct [33]. This change in specificity was probed further with *Synechococcus* 7002 rGln.

At neutral pH, the electronic absorption spectrum of ferric H46L *Synechococcus* 7002 rGln-R (Fig. S1) was consistent with coordination of a water molecule on the distal side. The ^1H NMR spectrum was in agreement as it exhibited the downfield hyperfine-shifted resonances of an aquomet complex (Fig. S2) and no strongly upfield shifted signals that could be attributed to the *meso* protons of a pentacoordinated species [34]. The pH dependence of the optical spectrum indicated a transition from this state at low pH to a hydroxymet species at high pH. At both extremes of pH, the complexity of the pH titration profile (Fig. S1) reflected aggregation, heme loss, and denaturation. The thermal denaturation profile monitored by electronic absorption spectroscopy lacked a native state

baseline (data not shown). Compared with ferric H46L *Synechocystis* 6803 rGlbN-R, which formed a His-Fe(III)-X complex [33, 35], the *Synechococcus* variant had reduced stability and different propensities for Fe(III) coordination.

Figure 2 presents the result of treating various forms of *Synechococcus* 7002 rGlbN-R with DT. In all cases, the iron was reoxidized with ferricyanide after DT incubation and the products were identified in the cyanomet state so as to compare species with identical His-Fe(III)-CN coordination. Figure 2, spectrum A contains the ^1H NMR spectrum of wild-type cyanomet rGlbN-A, which was obtained from starting material with His-Fe(III)-His coordination. Reaction occurred at the 2-vinyl [14], and this spectrum is used for reference. When the reaction was performed on the cyanomet state of wild-type rGlbN-R [starting material with His-Fe(III)-CN coordination], the resulting spectrum (Fig. 2, spectrum B) indicated the formation of a small amount of an additional product. When ferric H46L *Synechococcus* 7002 rGlbN-R was used as the starting material without added exogenous ligand [His-Fe(III)-OH₂/OH⁻], the second product was formed in a 2:1 ratio compared with the 2-vinyl adduct (Fig. 2, spectrum C). Analysis of homonuclear data and ^1H - ^{15}N HMQC data (Fig. S3) confirmed that this second species contained a covalent linkage to His117 N ϵ 2. Cyanomet H46L *Synechococcus* 7002 rGlbN-R [His-Fe(III)-CN] yielded products in an approximately 1:2.5 ratio in favor of the 2-vinyl adduct (Fig. 2, spectrum D).

Homonuclear and ^1H - ^{13}C HMQC data were used to assign the heme methyls in cyanomet H46L *Synechococcus* 7002 rGlbN-A² and rGlbN-A⁴ (where the superscript specifies the reacted vinyl). The chemical shifts are provided in Table S1 and sample data are shown in Fig. S4. The ^1H heme methyl order of the A² species was found to be 3 > 5 > 8 ~ 1 (from downfield up) and that of the A⁴ species was 3 ~ 8 >> 5 > 1. The angle between the N_A-N_C axis (Fig. 1a) and the projection of the proximal histidine imidazole plane onto the heme plane was estimated for each adduct [36]. The shifts of the A² species indicated that the projection of the imidazole plane aligned within a few degrees of the β - δ meso axis as in wild-type rGlbN-R [14]. This is a “staggered” orientation offering minimal steric clash between the porphyrin nitrogen atoms and the imidazole ring CHs. The shifts of the A⁴ species indicated that from its rGlbN-R orientation, the heme underwent a 180° rotation about the α - γ axis, followed by an in-plane rotation of approximately 40°, a rearrangement that brings the 4-vinyl within bonding distance of His117, but also aligns the projection of the proximal histidine imidazole with the porphyrin N_B-N_D axis. This is a strained “eclipsed” orientation [37] expected to impart distinct chemical properties to the two adducts from altered orbital overlap between the histidine nitrogen p_π orbital and the d_π orbital of the heme iron [38].

As observed in *Synechocystis* 6803 rGlbN [33], the replacement of the distal histidine therefore led to a loss in posttranslational modification specificity. In *Synechococcus* 7002 rGlbN, however, the effect was more pronounced, causing a reversal of vinyl preference upon H46L substitution. The 40° in-plane rotation of the heme locates a propionate group at the position occupied by the 8-CH₃ in wild-type rGlbN-R. We hypothesize that the steric hindrance at that site is less severe in *Synechococcus* 7002 rGlbN (Ser65) than in *Synechocystis* 6803 rGlbN (Tyr65), therefore facilitating the addition. Further speculation on the origin of the variable A² to A⁴ ratio is rendered difficult by the complexity of the overall reaction, which involves multiple competing processes, e.g., protein conformational changes, ligand decoordination, and heme reorientation.

Results obtained in the ferrous state in the presence of carbon monoxide provided an additional clue for the influence of the distal ligand on the reactivity of rGlbN-R. When solutions of *Synechocystis* 6803 rGlbN-R were first saturated with CO then reduced with DT, variable amounts of CO-bound rGlbN-A were obtained, which reflected a competition

between CO binding and cross-link formation in the His–Fe(II) or the His–Fe(II)–His state. Likewise, *Synechococcus* 7002 rGlbN-R reduced by DT in the presence of CO failed to convert to homogeneous CO-bound rGlbN-A samples. ¹H NMR spectra of a *Synechocystis* 6803 rGlbN-R–CO/rGlbN-A–CO mixture are presented in Fig. S5, with heme assignments in Table S2.

His46 coordination and H₂O₂ damage

Many cyanobacteria live under high oxidative stress and possess robust and redundant mechanisms to eliminate ROS and RNS. Heme proteins, unless dedicated to this detoxification, are particularly susceptible to ROS damage. Known mechanisms of resistance involve modes of iron coordination, heme pocket composition, and heme chemical modifications. The extent to which H₂O₂, a predominant cyanobacterial ROS, destroys rGlbN was therefore inspected. Figure 3 displays the results of treatment of ferric wild-type *Synechococcus* 7002 rGlbN-R with a fivefold excess of H₂O₂. Here the optical spectrum was used to detect heme bleaching. Immediately following H₂O₂ addition, there was no evidence for the formation of a hydroxyferryl complex (compound II) as seen in horse heart myoglobin [39, 40]. Over time, the Soret band intensity decreased as the heme was degraded. In contrast, when the H46L variant was used, rapid bleaching occurred. Cyanide binding prior to H₂O₂ treatment protected this protein from damage. Similar observations were made with *Synechocystis* 6803 GlbN and its H46L variant (Fig. S6).

Oxidative damage often manifests itself as intermolecular *o,o'*-dityrosine cross-links [41]. *Synechocystis* 6803 GlbN and *Synechococcus* 7002 GlbN possess five and three tyrosines, respectively, and are a priori capable of forming dimers and higher molecular weight cross-linked species. *Synechocystis* 6803 and *Synechococcus* 7002 rGlbN-R samples exposed to H₂O₂ were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The results are presented in Fig. 4 for *Synechococcus* 7002 rGlbN and in Fig. S7 for *Synechocystis* 6803 rGlbN. Regardless of amino acid replacement and cross-linking state, ferric samples treated with H₂O₂ contained species with twice and some thrice the protein molecular weight. Cyanide binding prior to H₂O₂ treatment inhibited oligomer formation presumably by blocking access to the heme iron. Endogenous coordination by His46 attenuated the damage, but under excess of H₂O₂ was not as effective as bound cyanide.

The *o,o'*-dityrosine linkage is readily identified by its fluorescence emission spectrum [42], provided that the heme group can be removed from the protein. Following acid–butanone extraction of the heme, the apoprotein samples were analyzed by fluorimetry. The results are particularly clear for *Synechocystis* 6803 GlbN because of higher tyrosine content and a higher proportion of oligomeric species with lower concomitant heme degradation. A large increase in fluorescence emission at 410 nm was observed after treatment with H₂O₂, confirming the nature of the product. The spectrum is shown in Fig. S8.

Protein-based radicals generated in the reaction with H₂O₂ may cause other modifications, for example, covalent heme attachment as mentioned earlier for ascorbate peroxidase [18]. In the preparation of samples for fluorescence analysis, the heme group was successfully extracted from wild-type *Synechocystis* 6803 rGlbN-R after a 3-h treatment with a fivefold excess of H₂O₂, indicating these reactions did not occur appreciably. In the case of wild-type *Synechococcus* 7002 rGlbN and, more obviously, its H46L variant, the aqueous phase retained some coloration after treatment. NMR data collected on the latter after addition of cyanide to the mixture (not shown) confirmed that the colored products were not rGlbN-A² or rGlbN-A⁴.

A proposed role for heme adducts in human peroxidases is the prevention of heme degradation by chemical reaction with reactive intermediates [43]. The ability of the heme–

protein cross-link to limit peroxide-induced heme degradation and radical formation was therefore examined. rGlbN-R and rGlbN-A showed comparable responses (Figs. 3, S6), demonstrating that the cross-link did not influence H₂O₂ reactivity significantly.

Reduction with DTT

Ferric rGlbN-R in solution undergoes the histidine–heme modification unpredictably and over days to months. Addition of DT accelerates the reaction such that wild-type samples are converted on a second to minute timescale at neutral pH. To determine whether the key feature of the DT reaction was the reduction of the heme iron or the involvement of the products of DT oxidation, DTT was used as an alternative reducing agent that produced distinct radical species and had a different mechanism of reaction with dioxygen [44]. The NMR spectrum of wild-type *Synechocystis* 6803 rGlbN-R incubated with a 20-fold excess of DTT, collected 20–30 min after addition, contained resonances corresponding to ferric rGlbN-A (Fig. 5). The spectrum also contained resonances from ferric rGlbN-R and additional broad and paramagnetically shifted peaks. As incubation continued, ferric rGlbN-R signals disappeared, while those of ferric rGlbN-A remained approximately at the same level. Addition of K₃[Fe(CN)₆] to the sample after 12 h converted the sample to the ferric state and only rGlbN-A signals were obtained. The same sequence of events occurred with samples of *Synechococcus* 7002 rGlbN-R. In this case, the broad signals attributed to the binding of DTT or its oxidation by-products to the ferric protein were stronger and disappeared only when the sample was passed through a Sephadex G-25 desalting column (Fig. S9).

The reaction was also examined by electronic absorption spectroscopy. *Synechococcus* 7002 rGlbN-R was chosen for this purpose because of the slightly larger difference between the spectra of rGlbN-R and rGlbN-A. Figure S10 shows the changes in the spectrum as a function of time after addition of 11-fold molar excess DTT in the presence of the oxygen-scavenging system GODCAT. At first, ferric rGlbN-R disappeared while ferric rGlbN-A accumulated. This corresponded to the reduction of the heme iron followed by cross-link formation and rapid reoxidation as the iron center catalyzed the elimination of residual dioxygen in solution [25, 45]. At later times, slow appearance of the ferrous state dominated the kinetic profile and corresponded to reduction opposed by reoxidation as dioxygen diffused into the imperfectly sealed sample cell. In addition to the reasons mentioned in the context of the H46L variant, the recorded kinetics were complicated by low but variable amounts of dioxygen present in the sample cell and, possibly, the expected decrease in the heme redox potential caused by the cross-link [46] allowing oxidation of ferrous rGlbN-A by ferric rGlbN-R. The meaningful results were that rGlbN-A was systematically obtained at both NMR and optical concentrations and that reduction was essential to the acceleration of cross-link formation.

Requirement for O₂ in the cross-linking reaction

In view of the aforementioned results, the need for O₂ in the posttranslational modification was inspected by performing the DT reduction under anaerobic conditions. The raw data for a representative experiment (36 μM ferric *Synechocystis* 6803 rGlbN-R, 1.25-fold excess DT, pH 8.2) are shown in Fig. 6a. In the first second of the reaction, the resolved Q bands of a ferrous species grew at 530 and 560 nm; these bands then decreased in intensity while bands developed at 526 and 558 nm. In this second phase, which was over in approximately 1 min, several isosbestic points were detected. Singular value decomposition of the time course followed by global fitting of the data to a sequential A → B → C mechanism returned residuals hinting at a more complex mechanism, likely involving low-amplitude rapid phases not well captured by the data collected at short times (200 ms to 1 s). Spectral changes occurring at longer times (*t* > 1 s) were consistent with a well-defined B → C step

proceeding with $k_{\text{obs}} \sim 0.1 \text{ s}^{-1}$. The spectra of the two reduced species are shown in Fig. 6b (solid lines). Species C was readily identified as ferrous rGlbN-A by comparison with known spectra.

The most noticeable differences between the spectrum of the reduced intermediate and that of ferrous rGlbN-A were a hypsochromic shift and the hypochromicity of the latter. The shift was consistent with saturation of one heme vinyl group. When ferric H117A *Synechocystis* 6803 rGlbN-R was used as the starting material in the stopped-flow experiments, reduction was achieved within 1 s and no further spectral change was observed. The spectrum of ferrous H117A *Synechocystis* 6803 rGlbN-R (Fig. 6b, dashed line) resembled closely that of the intermediate species and confirmed that the ferrous intermediate (B) was rGlbN-R.

The conversion of *Synechocystis* 6803 rGlbN-R to rGlbN-A was performed by manual mixing at pH 9.5 with various concentrations of DT and in the presence or absence of the GODCAT dioxygen scavenger. Reduction (A $\rightarrow$ B step) occurred during the dead time of the experiment (10–30 s). Several isosbestic points corresponding to the B $\rightarrow$ C step were observed (Fig. S11), and global analysis of the time traces returned a k_{obs} of approximately $3 \times 10^{-3} \text{ s}^{-1}$. Preliminary experiments showed this rate constant to be independent of DT or rGlbN concentration and to be approximately 40-fold slower than at pH 8.2. Performing the reaction in D₂O at a pD of 9.5 indicated a moderate solvent kinetic isotope effect of approximately 2.

Proposed mechanism of histidine–heme cross-link formation

Collectively, the data suggested a cross-linking mechanism (Scheme 1) by which the 2-vinyl group is protonated at the C β position to generate a C α carbocation that subsequently undergoes nucleophilic attack by a neutral His117. Reduction of the heme iron would accelerate the reaction by increasing the electron density on the 2-vinyl, and coordination of electron-withdrawing ligands, such as CO, would decelerate or inhibit the reaction. Two aspects of Scheme 1 were tested by inspecting the product obtained when the reaction was performed in D₂O or when a cysteine replaced the histidine at position 117.

Product obtained in D₂O

The carbocation mechanism includes the protonation of the 2-vinyl C β H₂. Performing the reaction in D₂O was therefore expected to produce a singly or multiply deuterated methyl at the C β position. For the purpose of identification, two-dimensional ¹H–¹³C correlation data were collected without ¹H decoupling in the indirect dimension. In this experiment, a CH₃ group gives rise to a 3:1:1:3 quartet, whereas a CH₂D group yields a 2:0:2 triplet and a CHD₂ group yields a doublet, both further split by deuterium coupling. Figure 7 shows the spectral region containing the 2-¹³C β ¹H₃ cross-peak in wild-type *Synechocystis* 6803 rGlbN-A [14]. A triplet, too broad for the deuterium coupling to be resolved, was obtained when the reaction was performed in D₂O. No quartet corresponding to a CH₃ group was detected. Under all chosen conditions, *R* stereospecificity was maintained at C α , and multiple exchange steps resulting in further deuteration of the vinyl group did not occur. In addition, the cross-peak from the 2-C α H was present with intensity comparable to that observed when the reaction was performed in H₂O. Thus, mechanisms involving nonlabile hydrogen abstraction were eliminated.

Reactivity of H117C rGlbN

His117 was replaced with a cysteine to test the ability of an alternative nucleophile to form the Markovnikov adduct to the 2-vinyl. The ¹H spectrum of ferric H117C *Synechocystis* 6803 rGlbN-R was consistent with a minimally perturbed bishistidine complex (Fig. 8,

spectrum A), as observed for the H117A variant [14]. Incubation of the ferric protein with DT followed by reoxidation resulted in an altered spectrum that indicated chemical modification of the heme (Fig. 8, spectrum B). The acid–butanone extraction procedure yielded a colored aqueous phase characteristic of a heme group covalently attached to the protein. The pyridine hemochrome spectrum of the product (Fig. S12) was within 1 nm of that of wild-type rGlbN-A and had an α band with λ_{max} at 554 nm, 3 nm lower than the expected 557 nm of a *b*-type heme [47] and comparable to that of the species obtained from a cysteine variant of cytochrome *b*₅ that contains a single thioether linkage [48].

¹H and ¹H–¹³C correlated NMR data (Fig. S13) were used to identify the signals of the modified heme. They confirmed that the four methyl groups were intact and that the 2-vinyl group had been eliminated. They also identified *J*-coupled signals at ¹H frequencies of –0.1 and 3.2 ppm arising from a porphyrin 2-substituent. A natural abundance ¹H–¹³C HSQC spectrum collected without decoupling in the indirect dimension revealed the 3:1:1:3 signature of a CH₃ group at –0.1 ppm (Fig. 9; heme chemical shifts are listed in Table S3). Thus, the modified heme contained a –CH–CH₃ moiety at the 2 position of the porphyrin ring.

Modeling Cys117 in the wild-type rGlbN-R structure [49] places the S γ atom at more than 3 Å from the heme 2-C β atom and at a greater average distance still from the heme 2-C α atom. Because a structural rearrangement is required for a cysteine to reach the heme 2-vinyl C α , and cysteines are more prone to radical formation than histidines, the absence of product containing a linkage at C β lent further support to Scheme 1.

When H117C *Synechococcus* 7002 rGlbN-R was used as the starting material for DT reduction, heme modification occurred as well (Fig. S14). However, the reactivity appeared to be lower than in H117C *Synechocystis* 6803 rGlbN-R, and samples of modified H117C *Synechococcus* 7002 rGlbN suitable for complete spectral analysis were not obtained. For this protein as well, the distance of closest approach between a modeled Cys117 S γ and the heme 2-vinyl C α exceeds 3 Å in the rGlbN-R and rGlbN-A structures. It is possible that the structural fluctuations that allowed Cys117 to reach the 2-vinyl group in *Synechocystis* 6803 rGlbN were less effective in *Synechococcus* 7002 rGlbN. Alternatively, the thiol group may be involved in different interactions in the two proteins.

As an additional test for a radical reaction involving compound I (an oxoferryl porphyrin π -cation radical) and a thiolate group [48], ferric H117C and H117A rGlbN-Rs were treated with H₂O₂ and their absorption spectra were examined. As in the wild-type proteins, bleaching was the principal consequence of incubation with fivefold excess of the peroxide. The spectral changes associated with the formation of rGlbN-A were not observed.

Propionates and vinyls in rGlbN-R

The stereoelectronic properties of the attacked vinyl depend on its orientation with respect to the porphyrin plane [50]. In the reduced state with CO bound, the wild-type rGlbNs have intra-heme nuclear Overhauser effects in agreement with the “*trans*” orientation of both vinyls: strong effect between the 2- α -H and the α -*meso* (4- α -H and β -*meso*), and medium-to-small effect between the 2- β -H₁ and 1-CH₃ (4- β -H₁ and 3-CH₃). The same holds for the ferric bishistidine states. The temperature response of the chemical shift provides additional information in these low-spin complexes [51]. A plot of 2-vinyl ¹H chemical shifts versus inverse temperature (Curie plot) is presented for both *Synechococcus* 7002 rGlbN-R and *Synechocystis* 6803 rGlbN-R in Fig. S15. The trends were linear over the narrow range of temperatures allowed in the experiment and indicated little mobility of the groups.

The electron density of the heme group can also be modulated by the interactions of the propionates with the protein [52–54]. In *Synechocystis* 6803 rGlbN [55, 56] and presumably in *Synechococcus* 7002 rGlbN as well, the 6-propionate is exposed to solvent, whereas the 7-propionate is engaged in electrostatic interactions and hydrogen bonds. These features are sensitive to the nature of the distal ligand to the heme [56]. Although a complete analysis of propionate interactions is beyond the scope of this report, it should be noted that in all complexes that have been inspected thus far by NMR spectroscopy [14], including cyanomet H46L *Synechococcus* 7002 rGlbN-A² (Fig. S4), the 6-propionate ¹³C α signal is systematically approximately 12 ppm upfield of the 7-propionate ¹³C α signal. Considering that the orientation of the proximal histidine is along the β - δ *meso* axis, this disparity in contact shift may be linked to a biased electronic distribution.

Discussion

Hemoglobins with bishistidine coordination have been the subject of studies seeking to establish their standing in the evolution of the hemoglobin superfamily [57–59] and to determine the implications of their coordination scheme [5, 6, 35, 60–62]. Human neuroglobin [63] and possibly α hemoglobin in complex with the chaperone α -hemoglobin-stabilizing protein [64] illustrate that blocking the distal site with a histidine can limit the reactivity of the heme group, protecting it from damage and eliminating side reactions generating ROS. Replacement of the distal histidine of *Synechocystis* 6803 rGlbN and *Synechococcus* 7002 rGlbN, whether rGlbN-R or rGlbN-A, with a weaker ligand resulted in increased H₂O₂ damage and confirmed that some measure of protection was conveyed by the bishistidine scheme.

Physiological data obtained on *Synechococcus* 7002 show that GlbN imparts substantial resistance to the oxidative/nitrosative stress caused by high nitrate concentrations as the source of nitrogen in the growth medium [8]. The attenuated susceptibility to damage provided by distal histidine coordination may therefore be advantageous to GlbN in the cyanobacterial cell. However, at least one other important consequence of this distal coordination was noted in vitro: without it, the posttranslational modification of the heme group yielded not one but two products that differed in the reacted vinyl group, the orientation of the heme in its cavity, and presumably chemical reactivity.

Cross-linking in *Synechocystis* 7002 and *Synechococcus* 6803 GlbNs may be necessary if, during their function, these proteins visit states of low heme affinity. This is expected of the ferrous protein [46] and in complexes with certain exogenous ligands (e.g., NO [65]). Interestingly, the levels of heme oxygenase 2 increase significantly under microaerobic conditions in *Synechocystis* 6803 [66, 67] and heme attachment would prevent heme degradation. The fact that Cys117 can add to the 2-vinyl efficiently, at least in *Synechocystis* 6803 rGlbN, confirmed that a histidine is not the only solution to the irreversible attachment of the heme to GlbN. Histidine is generally less reactive than cysteine and may be better suited if the protein must reside in a cellular environment where *S*-nitrosylation and other reactions could block the thiol functionality.

Regarding the mechanistic aspects of the posttranslational modification, the results obtained with H46L *Synechococcus* 7002 rGlbN-R indicated that a pentacoordinate ferrous state (or a ferrous state with loosely associated water) was reactive. The nature of the wild-type species undergoing the addition, however, remains undetermined. In the ferric state, we have found no evidence for the population of pentacoordinate wild-type rGlbN complexes, but reduction of the heme iron will weaken the distal histidine ligation bond [68, 69], and decoordination may occur. The optical spectra of ferrous H117A *Synechococcus* 7002 rGlbN-R and *Synechocystis* 6803 rGlbN-R were inspected using the empirical correlation between the

fractional population of the bishistidine complex and the ratio of the absorbance at 555 and 540 nm reported by Smagghe et al. [69]. The ratio was 2.3 for ferrous H117A *Synechocystis* 6803 rGlbN-R and only 1.6 for ferrous H117A *Synechococcus* 7002 rGlbN-R. These values correspond to approximately 100% and approximately 40% bishistidine hexacoordination, respectively, and it is possible that the same trend holds in wild-type rGlbN-R. The rates of His46 decoordination (14 s^{-1} [70]) and recoordination ($4,200 \text{ s}^{-1}$ [35]) in ferrous *Synechocystis* 6803 rGlbN-A are relatively fast. If similar rates apply in GlbN-R, the mechanism of cross-linking could include fast pre-equilibrium between hexacoordinated and pentacoordinated species. A pentacoordinate, His-Fe(II), reactive species may also be formed when cyanomet GlbN is reduced.

The reductive electrophilic addition shown in Scheme 1 was proposed for the biosynthesis of the histidine-heme linkage in the cytochrome *c* maturation protein CcmE [71] before it was determined that this linkage was of the $\text{Nd1}-\text{C}\beta$ type [16] rather than the $\text{Ne2}-\text{Ca}$ type as in GlbN. In vitro formation of thioether bonds in *c*-type cytochromes is also thought to be initiated by protonation of the vinyls [48, 72, 73], the reactive cysteines serving as proton donors. The evidence presented here for GlbN supports the nonradical electrophilic route for several reasons. rGlbN-A² (addition at *Ca*) is produced at the exclusion of other species under a large number of conditions, which include (1) anaerobic reduction with DT, (2) microaerobic (i.e., substoichiometric O₂) reduction with DTT, and (3) His117 replacement with cysteine. In addition, rGlbN-R did not yield rGlbN-A when H₂O₂ was added to the ferric state. Preliminary kinetic analysis demonstrated that proton transfer contributed to the rate-determining step. His117 has a pK_a of 6.9 in ferric *Synechocystis* 6803 rGlbN-R [14] and a pK_a of 6.1 in ferric *Synechococcus* 7002 rGlbN-R [8]. Assuming that a similar pK_a difference persists in the ferrous state, comparative rate measurements as a function of pH will aid in the identification of the proton donor. These experiments are in progress.

What are the factors that control the cross-linking reaction? A sine qua non condition is that the nucleophile and the vinyl group must be able to reach each other. The solution structures of *Synechocystis* 6803 rGlbN-R [49] and *Synechococcus* 7002 rGlbN-R [8] show that His117 points toward solvent most of the time, and that a change in rotameric state suffices to direct the imidazole ring to the 2-vinyl. Although the differences between the two rGlbNs were exacerbated with the shorter Cys117 side chain and suggested that a key structural or dynamic property of the nucleophile's local environment was important, the geometrical constraints on the nucleophile and vinyl *Ca* did not appear particularly difficult to satisfy. Schneider et al. [74] have inspected the three-dimensional structure of multiple *b* heme proteins and found variability in the composition of the heme edge, which emphasizes that cysteines and histidines can a priori be accommodated near vinyl groups.

The production of rGlbN-A² and rGlbN-A⁴ with identical $\text{Ne2}-\text{Ca}$ linkages starting from what must be states with different vinyl packing and heme environment argues for broad applicability of Scheme 1 to other heme proteins. Yet, this type of posttranslational modification is rarely observed, and additional determinants of reactivity are likely at work. The vinyl groups of rGlbN were ordinary in geometry (*trans* orientation) and mobility (linear Curie behavior) and did not stand out as candidates. However, subtle influences, such as heme distortions [75] and heme propionate interactions [53, 54], and less subtle influences, such as axial ligand nature, affect the heme electron density and can restrict the number of proteins and states in which the reaction is possible.

Not surprisingly, we have obtained evidence that oxidation and ligation state alter the reactivity of rGlbN. These two factors may explain the low levels of cross-linked GlbN in *Synechococcus* cells grown under aerobic conditions [8] because of either oxygen binding or iron oxidation. It is interesting that the heme group in the X-ray structures of *Synechocystis*

6803 rGlbN-A and H117A rGlbN shows out-of-plane distortions that differ from the doming typically observed in globins. Ruffling is pronounced in cyanomet and azidomet rGlbN-A, with magnitudes comparable to those in *c*-type cytochromes [75], and, as mentioned in “Results,” the interactions of the heme propionates with the protein are asymmetric. The consequences of these various features are not readily deconvoluted from the rGlbN data, but will be worth investigating experimentally and computationally with other rGlbN variants and modified hemes.

The two thioether bonds in *c*-type cytochromes are formed from a CXXCH motif, the role of which has been discussed extensively [73, 76, 77]. An XXXCH signature, leading to the formation of a single thioether bond, is recognizable in mitochondrial cytochromes *c* and *c*₁ from trypanosomatids and euglenids [78]. Single thioether bonds to heme are also found in certain *b₆f* cytochromes [79], but their sequence motif is less distinctive. In prior work [8], we provided a list of five additional group I 2/2 hemoglobins with high sequence homology to the GlbNs discussed here. These proteins are expected to exhibit bishistidine coordination and to undergo the histidine–heme modification. The latter inference is based on the presence of a histidine at an appropriate topological position and not on local H-helix sequence, which is quite variable. Whether a labile sixth axial ligand is an essential feature of proteins capable of the posttranslational modification remains to be explored, but in any case, no simple primary structure criterion is likely to emerge for the formation of histidine–heme bonds. Our results caution that chemical characterization will be required to ascertain bond formation even for proteins with high structural homology near heme vinyl groups, but they also suggest that the modification may be more common than currently thought.

In conclusion, we presented evidence that the histidine–heme covalent bond in reduced GlbN is formed by an electrophilic addition. We propose that a similar addition occurs in vitro in *c*-type cytochromes and in vivo in *Synechocystis* 6803 and *Synechococcus* 7002 cells. The ease with which various forms of rGlbN can be prepared will allow for a further examination of the reaction and the definition of the most important determinants of reactivity for this posttranslational modification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DT	Dithionite
DTT	Dithiothreitol
GODCAT	Glucose oxidase/D-glucose/catalase
HMQC	Heteronuclear multiple quantum coherence
HSQC	Heteronuclear single quantum coherence

rGlbN	Recombinant GlbN
rGlbN-A	Recombinant GlbN with covalently attached heme
rGlbN-R	Recombinant GlbN prepared by reconstitution of the apoprotein with iron(III) protoporphyrin IX
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
<i>Synechococcus</i> 7002	<i>Synechococcus</i> sp. PCC 7002
<i>Synechocystis</i> 6803	<i>Synechocystis</i> sp. PCC 6803

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Fig. 1.

a The structure of the modified heme with the nomenclature used in the text. The unmodified *b* heme has vinyl substituents at positions 2 (pyrrole B) and 4 (pyrrole C). **b** Ribbon diagram of *Synechococcus* sp. PCC 7002 (*Synechococcus* 7002) recombinant GlbN with covalently attached heme (rGlbN-A) (minimized average of NMR ensemble 2ksc). The axial histidines (His46, distal, and His70, proximal) are shown, along with the histidine (His117) forming the covalent adduct to the 2-vinyl. Helices are labeled A, B, C, E, F, G, H, and H'

Fig. 2.

^1H NMR spectrum of cyanomet *Synechococcus* 7002 rGlbN-A obtained by reduction of ferric rGlbN prepared by reconstitution of the apoprotein with iron(III) protoporphyrin IX (rGlbN-R) with dithionite (DT) followed by reoxidation and cyanide binding (down-field hyperfine-shifted region). **A** The wild-type protein in its bishistidine resting state reacted at the 2-vinyl position. Peaks *a* and *b* are heme methyl groups. **B** A small amount of an additional product is obtained when the wild-type protein is in the cyanomet state prior to DT treatment (resonances marked with *asterisks*). **C** The H46L variant reacted at both the 2-vinyl and the 4-vinyl positions, the adduct at position 4 being the major product. Peaks *c* and *d* are heme methyl groups. **D** The H46L variant in the cyanomet state prior to DT treatment reacted at both the 2-vinyl and the 4-vinyl positions, the adduct at position 2 being the major product. Data were collected in 10% D_2O , pH 7-7.5 and 25 °C at 600 MHz

Fig. 3.

Reaction of ferric *Synechococcus* 7002 rGlbN with H_2O_2 monitored by electronic absorption spectroscopy. The absorbance at the Soret band maximum is plotted as a function of time after addition of a fivefold excess of H_2O_2 . *Squares* wild-type rGlbN-R, *triangles* wild-type rGlbN-A, *diamonds* H46L rGlbN-R, *circles* cyanomet H46L rGlbN-R. The apparent signal recovery of H46L rGlbN-R after 20 min is attributed to scattering. The *solid lines* are drawn to guide the eye

Fig. 4.

Oligomerization caused by H_2O_2 treatment of *Synechococcus* 7002 rGlbN. Protein samples and conditions were as follows: *leftmost lane* molecular weight (*MW*) ladder; *lane 1* rGlbN-R, no treatment; *lane 2* rGlbN-R, 2-h incubation with fivefold excess H_2O_2 ; *lane 3* rGlbN-A, no treatment; *lane 4* rGlbN-A, 2-h incubation with fivefold excess H_2O_2 ; *lane 5* H46L rGlbN-R, no treatment; *lane 6* H46L rGlbN-R, 2-min incubation with fivefold excess H_2O_2 ; *lane 7* H46L rGlbN-R, 2-h incubation with fivefold excess H_2O_2 ; *lane 8* cyanomet H46L rGlbN-R, 3-h incubation with fivefold excess H_2O_2 . Note the presence of dimers in *lanes 2, 4, 6, and 7*

Fig. 5.

^1H one-dimensional NMR spectra of 1 mM wild-type *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803) rGlbN (600 MHz, 20 mM phosphate buffer pH 7.2, 25 °C, 10% D_2O). *A* Before dithiothreitol (DTT) addition, the protein is in the ferric rGlbN-R state. *B* 30 min after 20 mM DTT addition, ferric rGlbN-R signals have almost disappeared (indicated by *dashed lines* from *A* to *B*) and most of the protein is either in the reduced or in the oxidized rGlbN-A state. Note the presence of broad signals attributed to protein containing bound DTT species. *C* After overnight incubation, the protein is entirely in the ferric oxidized rGlbN-A state. Small differences in chemical shifts are due to changing conditions during the reaction

Fig. 6.

a UV-vis spectral results of rapid mixing of 36 μM wild-type *Synechocystis* 6803 rGlbN-R and 45 μM DT under anaerobic conditions. **b** Singular value decomposition of the data retrieved the spectra of ferrous rGlbN-R (B, black line, intermediate) and ferrous rGlbN-A (C, gray line, final product). The dashed line (D) shows the spectrum of ferrous H117A *Synechocystis* 6803 rGlbN for comparison with GlbN-R

Fig. 7. ^1H - ^{13}C correlated data collected on wild-type *Synechocystis* 6803 rGlbN-A produced by DT reduction of GlbN-R in D_2O . A region of the heteronuclear single quantum coherence (HSQC) spectrum is presented showing the location of the 2- $\text{C}\beta$ cross-peak. The *inset* contains the same in the absence of ^1H or ^2H decoupling in the ^{13}C dimension. The splitting pattern indicates the formation of a CH_2D group. Data were collected in 99% D_2O , 20 mM phosphate, pH 7.2 and 40 $^\circ\text{C}$ at 600 MHz

Fig. 8.

^1H one-dimensional spectra of H117C *Synechocystis* 6803 rGlbN in the ferric bishistidine state. *A* rGlbN-R, which resembles the wild-type spectrum, and *B* rGlbN-A, obtained after treatment of rGlbN-R with DT and reoxidation of the iron. The changes in the spectrum indicated that a modification had occurred. Data were collected at 600 MHz in 90% H_2O /10% D_2O , 20 mM phosphate, pH 8.7 and 25 °C. Labeled peaks are *a* 5- CH_3 , *b* 1- CH_3 , *c* 4-vinyl $\text{H}\beta$ *trans*, and *d* 4-vinyl $\text{H}\beta$ *cis*. Spectrum *A* contains a small amount of the minor heme orientational isomer, which nearly disappeared with further equilibration

Fig. 9. ^1H - ^{13}C correlated data collected on H117C *Synechocystis* 6803 rGlbN-A in the ferric bishistidine state. A portion of the HSQC spectrum is shown, which contains the cross-peak of the heme 2-C β . The *inset* contains the same in the absence of decoupling in the ^{13}C dimension. The multiplet structure identifies a methyl group. Data were collected in 99% D_2O , 20 mM phosphate, pH 8.7 and 25 °C at 500 MHz (decoupled data) and 600 MHz (*inset*)

Scheme 1.
Proposed cross-linking mechanism