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Novel Histidine−Heme Covalent Linkage in a Hemoglobin

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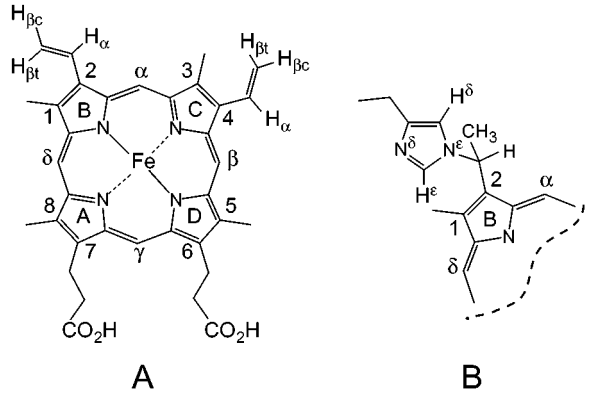
Over the past decade, a number of hemoglobins have been discovered in prokaryotes. The function of these globins of ancient origin is not well understood, but it is likely that some play catalytic and stress-response roles,[1] harboring unsuspected aspects of contemporary heme chemistry. The genome of the cyanobacterium *Synechocystis* sp. PCC 6803 (S6803) contains a single hemoglobin gene.[2] Protein expression levels in vivo are currently unknown. However, overexpression in *Escherichia coli* can result in the formation of inclusion bodies from which the 123-residue apoprotein (rHb) is readily purified. S6803 rHb binds one ferric heme group per polypeptide chain via two axial histidines to yield a hexacoordinate, low-spin holoprotein (rHb-R).[3−5] During solution NMR experiments, S6803 rHb-R was observed to convert spontaneously to an alternative form, rHb-A.[5] The heme group of this species was not extractable using the acid−butanone procedure effective with *b*-hemoproteins, and the hemochromogen assay pointed to a modification of a heme vinyl substituent.[6] Here, a combination of NMR and mass spectral methods was applied to characterize the heme−protein cross-link. The facile formation of a covalent adduct in a globin is unusual and suggests nontraditional roles for the hemoglobin in the cyanobacterium.

When rHb-A was first observed, the conditions promoting the covalent attachment of the heme group were not identified. In subsequent experiments aiming to produce the ferrous protein from the ferric form, it was found that treatment with a 1.5-fold excess of dithionite, a reagent typically used for this purpose, followed by gel filtration under aerobic conditions led to complete conversion of rHb-R to rHb-A at neutral pH. In this procedure, the color of the sample indicated reduction of the iron and rapid return to the oxidized state. Ferric rHb-A has optical properties similar to those of rHb-R.[6] In contrast, covalent heme attachment has a marked effect on the position of the hyperfine-shifted signals in the ^{1}H NMR spectrum of the protein. The *b* heme group (Scheme 1A) gives rise to distinctive scalar and dipolar connectivities generally used for ^{1}H NMR assignment. TOCSY, 2QF-COSY, and NOESY data were collected on S6803 rHb-A, and the signals from the four heme methyl substituents were readily assigned, as they were in S6803 rHb-R.[5] The AMX signals of the heme 4-vinyl group were found, but no peaks were detected that could be attributed to the 2-vinyl group. Instead, a strong NOE was observed between the heme 1-CH_3 and a resonance at 0.57 ppm at 32 °C. This signal was scalarly coupled to a 2.24-ppm resonance, and these two signals lacked further *J*-connectivities to protein protons, suggesting they originated from a modified heme 2-vinyl group.

To identify the protein residue responsible for the reaction with the 2-vinyl, rHb-A was subjected to mass spectral analysis after pepsin digestion at pH 2, 37 °C, for 24 h. Several fragments were generated, one of which contained the attached heme as determined

Scheme 1



by reversed phase HPLC−UV at 394 nm (M_r = 1848.9 Da, monoisotopic by ESI-MS). This fragment was further analyzed by collision-induced dissociation and confirmed to encompass 12 residues, ^{111}VAGAPAHKRDVL, at the C-terminal end of the protein. However, this experiment did not single out the reacting residue because the heme−protein linkage was too fragile to withstand collisional activation. The sequence of the fragment offered four candidates for reaction: His117, Lys118, Arg119, or Asp120. In *Synechococcus* sp. PCC 7002, which also undergoes heme attachment,[6] this short stretch of sequence is ^{117}HRND. The assumption that the same residue reacts in both proteins selected a histidine or aspartic acid as partner to the heme 2-vinyl group. The mass spectrometry results further favored the histidine for its potential to make labile adducts.

To test for the existence of a C−N linkage, ^{1}H−^{15}N HMQC data sets were collected on uniformly ^{15}N-labeled S6803 rHb-A. One such spectrum is shown in Figure 1. In this experiment, unmodified histidines give rise to, at most, two cross-peaks per ring nitrogen, corresponding to Nδ−CδH, Nδ−CϵH, Nϵ−CδH, and Nϵ−CϵH connectivities.[7] The CδH and CϵH ^{1}H signals of each histidine not coordinated to the iron ion were assigned by using homonuclear data and by analogy with rHb-R;[8] these proton assignments led to those of the ^{15}N signals in the HMQC spectrum. His117 displayed an unusual downfield shift for one of the nitrogen atoms, identified as Nϵ through its CδH and CϵH cross-peaks. Strikingly, a third cross-peak was detected for this Nϵ atom, to a proton resonating at 0.57 ppm at 32 °C. This indicated a new Nϵ−C covalent connectivity, in agreement with the formation of a bond with the heme 2-vinyl. In this model, the ^{15}N chemical shifts of the Nϵ-alkylated His117 are expected to be under the influence of the paramagnetic heme derivative. Scheme 1B reconciles the new bond, the detection of the 0.57−2.24 ppm spin system spatially close to the heme 1-CH_3, the mass spectral data, and the hemochromogen results with a Nϵ−CαH−CβH$_3$ moiety resembling the Sγ−CαH−CβH$_3$ linkage found in *c* cytochromes.

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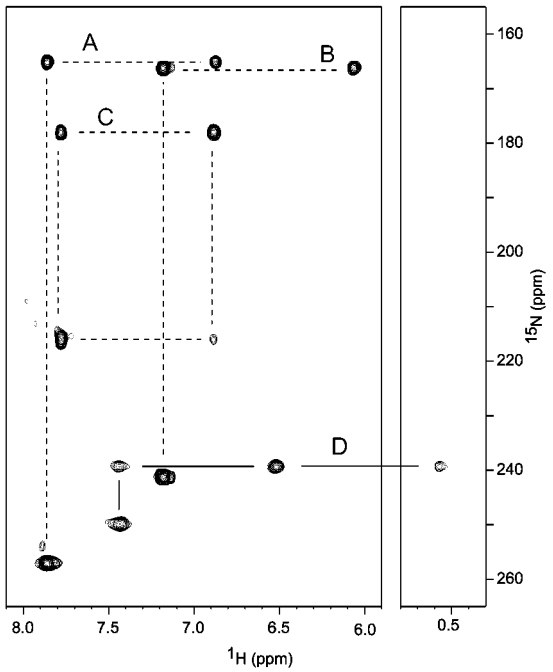

Figure 1. ^{1}H−^{15}N HMQC data for ^{15}N S6803 rHb-A in the ferric state. The spectrum was collected at 600 MHz at a protein concentration of ∼1 mM on a per-heme basis. The solvent was $^{2}H_2O$, buffered at pH* 7.1 with 20 mM phosphate; the probe temperature was 32 °C. (A) His83 and (B) His33 (both in the NϵH tautomeric state), (C) His77 (undergoing titration), (D) His117. This histidine has CδH at 6.52 ppm, CϵH at 7.43 ppm, Nδ at 250 ppm, and Nϵ at 239 ppm; note the additional connectivity between Nϵ and a carbon-bound proton signal at 0.57 ppm, assigned to the modified heme 2-vinyl.

Compared to the chemical shifts of rHb-R,[8] minor changes were observed in rHb-A for the residues remote from the heme. Larger-than-average deviations were experienced by the two axial histidines[9] and in the vicinity of heme pyrrole B.[6] In rHb-A, His117 CϵH is in dipolar contact with the heme 1-CH_3 and His117 CδH with the ring of Phe84. These interactions, which were not observed in rHb-R,[6] supported an imidazole ring orientation as in Scheme 1B and a conformational change at the C-terminal end of the protein upon reaction.

As expected if the 2-vinyl group underwent addition, the dithionite treatment of S6803 rHb reconstituted with 2,4-dimethyl deuterohemin resulted in the reduction of the ferric ion followed by a return to an unchanged ferric NMR spectrum. Dithionite treatment of S6803 rHb reconstituted with Zn protoporphyrin IX in the dark failed to produce a modified 2-vinyl species, whereas the same procedure applied to Fe(III) protoporphyrin IX resulted in complete conversion to rHb-A. This indicated that rapid reaction was independent of exposure to light and possibly that Fe(II) is more effective than Fe(III) in promoting the reaction. As with thioether linkages and the His-Tyr cross-link in cytochrome *c* oxidase, radical mechanisms are conceivable.[10] In this instance, the reaction may proceed via the species generated by oxidation of dithionite upon Fe(III) reduction. Regardless of the mechanism, cross-link formation must be considered in biophysical examinations of this hemoglobin, in particular if dithionite is used.

Well-documented linkages bridging a heme peripheral substituent to a protein side chain involve cysteine, methionine, aspartic acid, and glutamic acid.[11] These cross-links are generated with specialized enzymatic machinery, spontaneous reactions, or autocatalytic processes. Heterologous preparations of S6803 rHb from soluble *E. coli* cell extracts, in which a fraction of the isolated material is in the holoprotein state, were found to contain rHb-A.[3] Thus, the conversion may occur partially or completely in vivo as well. Interestingly, the primary structures of related globins show high variability at the C-terminal end,[1] but S6803 Hb and *Synechocystis* sp. PCC 7002 Hb are unique in having a histidine at or near position 117. The cross-link may play a functional role particular to these cyanobacteria, for example to increase the stability of the holoprotein, as proposed for cytochrome *c*,[12] or to prevent heme loss. Other effects will merit consideration, including the modulation of heme reactivity and, if adduct formation is not systematically achieved as a posttranslational modification or is reversible in vivo, participation in processes dependent on the conformational changes caused by heme attachment.

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Supporting Information Available: Experimental details for the preparation of rHb-R and rHb-A, heme, and axial histidines chemical shifts in rHbA, 1D traces of rHb-A and rHb-R, NOESY and TOCSY data used to assign the heme resonances, WEFT data, mass spectra, and HPLC-DAD analyses (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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