Biochemistry **1996,** *35,* 13627−13635

Bis-Methionine Ligation to Heme Iron in Mutants of Cytochrome b_{562}. 2. Characterization by NMR of Heme−Ligand Interactions[†]

Paul D. Barker*,[‡] and Stefan M. V. Freund[§]

Centre for Protein Engineering, MRC Centre, Hills Road, Cambridge CB2 2QH, U.K., and MRC Unit for Protein Function and Design, University Chemical Laboratory, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, U.K.

Received May 13, 1996[⊗]

ABSTRACT: Previous work has shown that, in variants of cytochrome b_{562} containing the H102M mutation, methionine residues provide both axial ligands to the heme iron. NMR spectroscopic studies of such bis-methionine-coordinated cytochrome have not previously been feasible, since the only other cytochrome with such a ligand arrangement, bacterioferritin, is too large to be studied by current NMR methods. The present work provides the first NMR characterization of 6-coordinate, bis-methionine-ligated heme centers in both ferrous and ferric oxidation states. We have used one and two dimensional, homonuclear NMR spectroscopy to assign the proton resonances of the heme group and ligand side chains in the reduced, cytochrome b_{562} variants, H102M and covR98C/H102M. The latter protein has heme covalently attached to the protein, and our results prove that the covalent linkage is a *c*-type thioether bond formed between the cysteine at residue 98 and the heme 2-vinyl group. Spectra of the ferrous H102M variant are consistent with the presence of two species differing in the orientation of the heme in the protein. We have interpreted results from NOESY experiments on the ferrous covR98C/H102M protein in terms of the conformation of the two methionine side chains, and we present a model for the structure of the heme ligand arrangement. The Met7 side chain adopts an extended conformation almost identical to that observed in the wild type protein with *R* stereochemistry at the chiral sulfur ligand. The Met102 side chain has a different, buckled side chain conformation and has *S* stereochemistry at the chiral center. Our NMR derived model is consistent with the spectroscopic data presented in the previous paper. Studies on the ferric forms of these proteins confirm that the double variant at low pH has a "stable" bis-methionine ligation arrangement, but that it is a thermal mixture of species with differing spin states. No hyperfine coupled proton resonances can be identified in spectra of the high-spin forms of either of these proteins.

The properties of a given heme protein can be significantly altered by changing the ligands to the heme iron. Yet comparison of the properties of different heme proteins which share the same nominal ligation arrangement continues to highlight the many subtle factors which can dramatically affect reduction/oxidation equilibria in these systems. While characterizing mutants of cytochrome b_{562} containing methionines at both of the residue positions which, in the wild type protein, provide the ligands to the heme iron, we have identified a new case in point (Barker et al., 1996). We can also generate cytochrome b_{562} proteins which have heme covalently attached to the protein through *c*-type, thioether linkages (Barker et al., 1995, 1996). Hence we characterized two cytochrome b_{562} proteins with bis-methionine ligation: one with covalently attached heme (covR98C/H102M) and one without (H102M). We showed that these proteins exhibit both oxidation state-dependent and pH-dependent electronic properties consistent with changes in coordination state. In their reduced states, both proteins have spectroscopic properties characteristic of low-spin, 6-coordinate ferrous heme centers. In their oxidized states, both proteins can exist in a high-spin state with properties consistent with the presence of only one methionine ligand, but the coordination state of the double variant, covR98C/H102M, is pH dependent. It undergoes a low-spin to high-spin transition with a pK_a of 7.1, and under conditions of low solution pH, this protein has bis-methionine ligation in both oxidation states. Consequently, its electron transfer reactions were uncomplicated by coordination state changes, and we were able to measure the reduction potential for the bis-methionine ligated heme group. Varying from +440 mV at pH 4.8 to +340 mV at pH 8.9, the reduction potential of this variant of cytochrome b_{562} is ~600 mV higher than that of bacterioferritin, which is the only other cytochrome to date known to have bis-methionine ligation to the heme iron (Cheesman et al., 1990; Frolow et al., 1994). The EPR spectrum of the ferric, bis-methionine-liganded covR98C/H102M protein (Barker et al., 1996) is unprecedented and is indicative of an electronic structure more axial in nature than the rhombic signal observed from the bacterioferritins. This suggested that the cytochrome mutant has a significantly different arrangement of the two methionine ligands than the pseudo-twofold symmetry arrangement found in bacterioferritin. In bis-histidine-coordinated cytochromes, the orientations of the two imidazole rings relative both to the heme plane and to each other are parameters thought to be a major factor in controlling heme electronic structure and properties (Timkovich, 1979; Moore & Pettigrew, 1990; Lee et al., 1993). In Met-His coordinated cytochromes, as well

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[†] This work was funded by ZENECA Group plc, the Medical Research Council, and the DTI under a Protein Engineering LINK program.

[*] Author to whom correspondence should be addressed; e.mail: pxb@mrc-lmb.cam.ac.uk.

[‡] MRC Center.

[§] University of Cambridge.

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1996.

S0006-2960(96)01128-2 CCC: $12.00

as the imidazole ring orientation, the orientation of the methionine side chain is thought to modulate properties, and some attention has focused on the stereochemistry at the methionine sulfur atom which is a chiral center when ligated to the iron (Senn & Wüthrich, 1985; Santos & Turner, 1993; Costa et al., 1994). Clearly, the conformations of the methionine side chains are the only variables in bis-methionine-ligated cytochromes, and this issue has not been addressed before now.

Here we present results from NMR spectroscopic studies of the proteins described previously (Barker et al., 1996). Results obtained from the diamagnetic, reduced proteins prove that, in the ferrous oxidation state, both methionines are ligated to the iron. We also present data proving the presence of the *c*-type thioether linkage between heme and protein in the variant with covalently attached heme. The results from homonuclear NOESY experiments have enabled us to identify the conformation and stereochemistry of each of the methionine ligand side chains in the reduced, covalently attached protein. We also present preliminary analysis of the proton NMR spectra of the paramagnetic, oxidized proteins and relate this information to our understanding of the electronic structure of these proteins. The structural information we have obtained can be related to the known structure and properties of bacterioferritin in order to establish the origins of the significant functional difference between very similar heme centers.

MATERIALS AND METHODS

The two proteins, H102M and covR98C/H102M, were expressed, isolated, and characterized as described (Barker et al., 1996). Protein samples (between 1 and 4 mM final concentration) were exchanged by ultrafiltration into 0.1 M phosphate buffers containing 0.5 M potassium or sodium chloride in D_2O (Sigma). The pH* of the solutions (uncorrected meter reading) was adjusted to values from 4.5 to 8.5. H102M was exchanged as the apoprotein followed by addition of 0.9 equivalents of bovine hemin (Sigma) dissolved in 0.1 M NaOD and re-equilibration of the sample to the correct pH*. D_3PO_4 and NaOD solutions in D_2O were obtained from Aldrich. Samples of reduced protein contained up to 10 mM sodium dithionite, while samples of oxidized proteins contained up to 20 mM potassium ferricyanide to prevent autoreduction which is a particular problem with the covR98C/H102M variant.

^{1}H NMR spectra of the reduced proteins were recorded on a Bruker AMX 500 MHz spectrometer at 300 K unless otherwise stated; NOESY and clean-TOCSY experiments were performed as previously described (Barker et al., 1995). One dimensional NMR spectra of the paramagnetic, ferric proteins were collected on either Bruker AMX 500 MHz or DRX 300 MHz spectrometers with standard Bruker pulse sequences which included presaturation of the residual solvent signal. Recycle delay times between 0.15 and 1.5 s were initially used to investigate the ^{1}H relaxation behavior. All experiments were then acquired with a 0.15 s repetition rate as 1024 transients of 4K data points with a sweep width of 72 kHz (145 ppm). Prior to Fourier transformation, free induction decays were apodized with a 10 Hz line broadening and zero filled to 16K. Nonselective T_1 measurements (Wu et al., 1991; La Mar & de Ropp, 1993) were made by the inversion recovery method (Vold et al., 1968). Chemical shifts for all spectra are referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate) via the residual solvent signal.

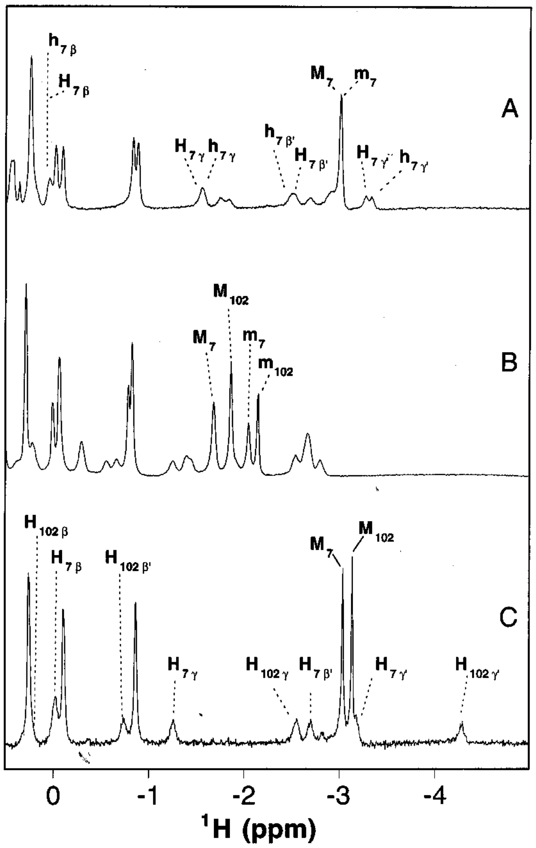

FIGURE 1: High-field region of the 500 MHz ^{1}H NMR spectra of (A) wild type (2 mM), (B) H102M (4 mM), and (C) covR98C/H102M (1 mM) ferrocytochromes b_{562} at pH* 6.6. Samples in (A) and (B) were obtained approximately 1 h after addition of 0.9 equivalents of hemin and sodium dithionite to 10 mM, both in the same buffer. Signals marked have been assigned to the methyl (M) and methylene (H) protons of the methionine ligands to the heme iron in each protein. Lower case m and h refer to the protons from species with the heme bound in the alternative orientation.

RESULTS

NMR Spectroscopy of the Ferrous Proteins. 1D 500 MHz ^{1}H NMR spectra of both H102M-containing holoproteins at pH* 6.6 are shown in Figure 1, along with that of the wild type protein for comparison. The regions shown contain resonances upfield of the main protein envelope where resonances from heme iron ligand protons are usually found, characteristically shifted by the large heme ring current. In the wild type protein (Figure 1A), the resonances labeled have been assigned to the protons of the Met7 side chain. This residue provides one axial ligand to the heme iron in each of two protein species which differ only in the orientation of bound heme about the $\alpha-\gamma$ heme axis (Wu et al., 1991; Barker et al., 1995). The Met7 methyl resonances (M_7, m_7) arising from these two species are degenerate and appear as one signal.

In contrast, four discrete methyl resonances (M_7, m_7, M_{102}, m_{102}) can be assigned to ring-current-shifted methyl groups of methionine axial ligands in spectra of the single mutant, H102M (Figure 1B). The ratios of these methyl resonances varied with time in a pairwise manner (M_7/M_{102} and $m_7/$

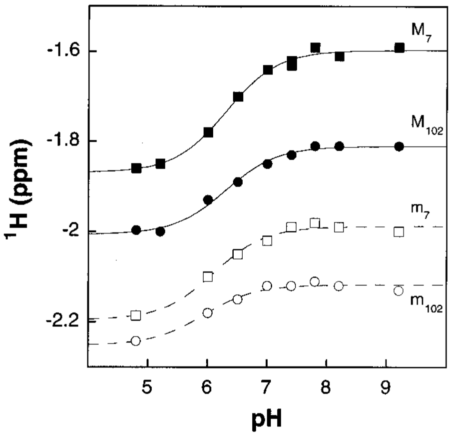

FIGURE 2: The chemical shifts of the methionine ligand ϵCH$_3$ protons in H102M as a function of pH*. Filled squares: Met7 major isomer (M$_7$ in Figure 1); filled circles: Met102 major isomer (M$_{102}$ in Figure 1); open squares: Met7 minor isomer (m$_7$ in Figure 1); open circles: Met102 minor isomer (m$_{102}$ in Figure 1). Solution was 4 mM protein loaded to 0.7 equivalents with heme and contained 0.5 M potassium chloride in D_2O. pH* adjustments were made by addition of 1 M D_3PO_4 and NaOD. Curves describe the best fits to a titration function involving one titratable group.

m$_{102}$ were each always in a 1:1 ratio) following addition of heme. These resonances have therefore been assigned to the ϵCH$_3$ groups of two methionine axial ligands in each of two heme orientations.[1] The sets of methionine protons were assigned to either residue 7 or 102 on the basis of results from NOESY spectra. Particularly, the methionine methyl resonance of residue 7 can be assigned, in each of the two overlapping spectra, on the basis of strong NOE's observed to the Phe65(3,5) and Phe65(4) proton resonances and other protons known to be on the Met7 side of the heme (data not shown, Barker et al., 1995).

The chemical shifts of these methionine methyl resonances are pH* dependent as shown in Figure 2. The dependencies on pH* of the chemical shift of these methyl resonances are consistent with the presence of a single titratable group, with a mean pK_a (in D_2O) of 6.3 in the major isomer and 6.0 in the minor isomer. This is in contrast to the dependence on pH of the optical spectrum of this species (Figure 1C,D of the preceding paper) which involves two titratable groups. Only the resonances from the equivalent protons in the minor isomer show any signs, at high pH*, of dependence on an additional titratable group. The analysis is complicated by the variance with pH* of the ratio of the isomers. Since we have not established that the heme orientation equilibrium was reached at each pH*, we cannot describe this effect further.

The spectrum of the double variant, R98C/H102M, is that of a single speices, and the heme is therefore present in only one orientation in this protein (*vide infra*). Two ring-current-shifted methionine ϵCH$_3$ resonances are observed (M$_7$ and M$_{102}$, Figure 1C), and their chemical shift values are independent of pH*. These resonances, and those from the associated methionine side chain spin systems (also labeled in Figure 1C), can be assigned to the two ligand residues 7 and 102. The two sets of methionine protons were again differentiated on the basis of strong NOEs observed between the Met7 methyl protons (δ -3.03 ppm) and the Phe65(3,5) and Phe65(4) protons and other protons known to be on the Met7 side of the heme (Barker et al., 1995).

The complete spectra of these two H102M-containing variant proteins can be analyzed in terms of two species being present in the single mutant and one in the covalent, double mutant, each with two methionines ligated to the heme iron. Results from TOCSY and NOESY experiments were used to assign the resonances of the heme group, the methionine ligand side chains, and the four aromatic group side chains. The proton chemical shifts of the relevant groups in both single and double variants are given in Tables 1S and 2S of the supporting information.

In the case of covR98C/H102M, TOCSY and NOESY experiments (Figure 3) provide proof that the heme is covalently linked to the protein through a *c*-type thioether linkage resulting from electrophilic addition of the Cys98 thiol to the 2-vinyl group. In these spectra the 2-vinyl group protons, present in spectra of wild type and H102M proteins, were missing. Instead, a single methine proton (δ 6.18 ppm) is correlated in the TOCSY spectrum to an additional methyl group (δ 2.28 ppm) (Figure 3A) which defines the -CHCH$_3$ unit of the thioether linkage. The strong NOESY peaks between the methine proton and the α-*meso* proton (δ 9.50 ppm, Figure 3B) prove that this thioether group is at the 2-position of the heme group. The NOE pattern between this -CHCH$_3$ unit and the α-*meso* and 1-methyl groups suggests that the stereochemistry at the chiral thioether sulfur atom is *S* as previously found for the single variant, R98C (Barker et al., 1995). The assignment of the 4-vinyl group was hampered by the chemical shift position of the *trans*-4β-vinyl proton which was found very close to the position of the residual solvent signal. As expected, due to the attenuation of these signals caused by the solvent presaturation, only weak crosspeaks between the 4α-vinyl proton (δ 8.29 ppm) and the *trans*-4β-vinyl (δ 4.80 ppm) were observed in the TOCSY experiment. The NOESY experiments confirmed these assignments, but again only very weak crosspeaks are observed. The upfield position of the *trans* proton can be explained based on its proximity to the aromatic ring of Phe65. This has previously been observed in other variants[2] (Barker et al., 1995). NOE's from the 4-vinyl and β-*meso* heme protons to the aromatic protons of Phe61 and between the heme 5-methyl and tyrosine aromatic protons (Figure 3B) confirm the orientation of the heme in the protein as that observed in the major isomer in the wild type and H102M proteins.

With four overlapping sets of methionine ligand side chain protons observed in the H102M protein, interpretation of the

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[1] To facilitate the assignment of the relevant protons of the two species observed in the spectrum of H102M shown in Figure 1B, TOCSY and NOESY experiments were performed on protein samples which contained different proportions of the species. Samples allowed to reach equilibrium in the oxidized state were a 6:1 mix of the two species upon reduction and immediate analysis. Equimolar mixtures were formed by reconstituting the holoprotein protein under strongly reducing and anaerobic conditions. With the wild type protein, this procedure yields a 1:1 mixture of two species, differing in the orientation of the heme about the $\alpha-\gamma$ heme axis, which are kinetically trapped due to a very slow rate of reorientation of the heme in the reduced protein. The spectra of the H102M protein 30 min after reconstitution show that two species are present as a 6:4 mixture. After 24 h the ratio was measured as 7:3. The changes in the spectra have not been followed to equilibrium. Analysis of NOESY spectra of this single mutant strongly suggests that, as in the wild type protein, the two species differ primarily in the orientation of the heme about the heme $\alpha-\gamma$ axis.

[2] This effect is also apparent for the *trans*-4-β-vinyl proton in the major isomer of the single mutant, H102M, and also all of the 2-vinyl protons in the minor isomer of H102M.

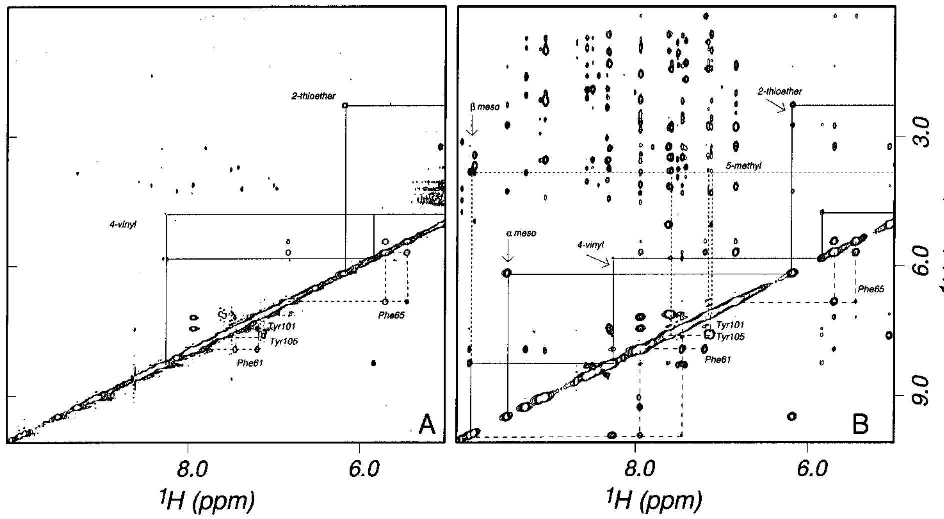

FIGURE 3: Downfield sections of 500 MHz ^1H TOCSY (A) and NOESY (B) spectra of reduced covR98C/H102M cytochrome b_{562} (1.5 mM in D_2O containing 0.1 M sodium phosphate and 0.5 M sodium chloride at pH*6.6). (A) In the TOCSY experiment through-bond connectivity between heme substituent protons is established by the labeled crosspeaks above the diagonal, while those crosspeaks defining the aromatic spin systems are shown below the diagonal. (B) Spin systems identified in (A) are connected to the heme α- and *β-meso* protons, which define the substituent position on the heme and the heme orientation. Also apparent, especially in the NOESY spectrum, are many crosspeaks involving amide protons which are very slow to exchange with the solvent. We have found this to be characteristic of the covalently-linked variants of cytochrome b_{562} which take months to completely exchange (Barker et al., 1995).

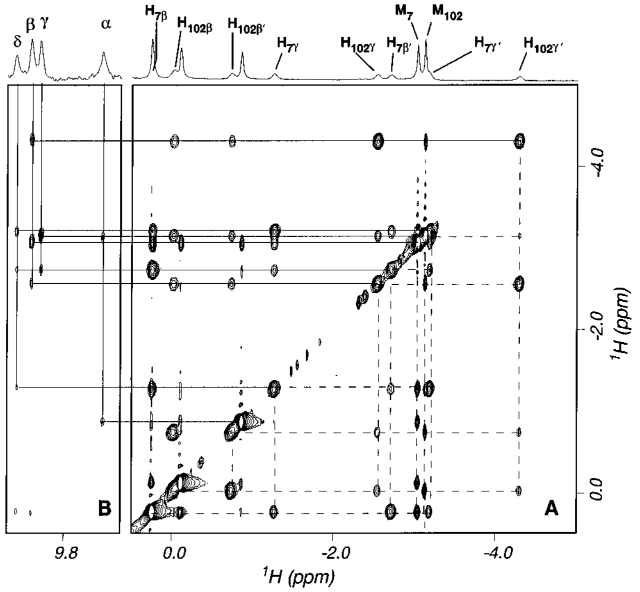

FIGURE 4: Sections of a 500 MHz ^1H NOESY spectrum of reduced, covR98C/H102M cytochrome b_{562} in the upfield (A) and *meso* proton (B) regions showing connectivity between the methionine ligand side chain protons and the heme *meso* protons. Protein was 2.5 mM in D_2O containing 0.1 M sodium phosphate and 0.5 M sodium chloride at pH* 6.6.

NOESY connectivity patterns for each methionine ligand is not straightforward. However, it should be noted that both the chemical shift values and NOESY connectivities of the methionine side chain protons are significantly different from those observed in either the wild type spectra (Met7) or the double mutant spectra (Met7 and Met102). For the double mutant, NOEs observed between the methionine ligand protons and heme group protons are illustrated in Figure 4. Figure 4A highlights the inter-side chain NOE's of the methionine ligands, and Figure 4B shows crosspeaks between heme *meso* protons and the methionine-ligand sidechains. The relevant contacts are listed in Table 3S of the supporting information.

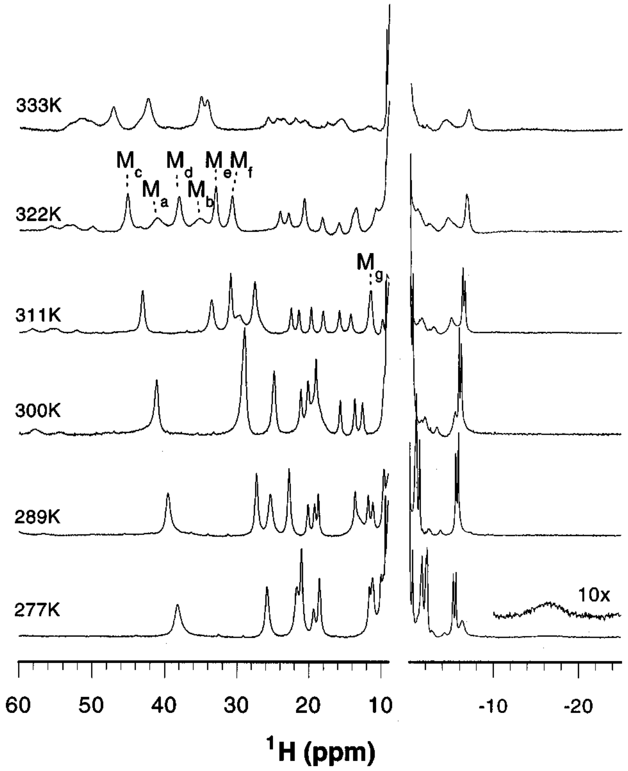

FIGURE 5: Temperature dependence of the 500 MHz ^1H spectrum of oxidized covR98C/H102M cytochrome b_{562} at pH* 4.5. Temperatures in Kelvin as indicated. Protein concentration was 2.4 mM.

NMR Spectroscopy of the Ferric Proteins. 1D 500 MHz ^1H NMR spectra of oxidized covR98C/H102M at pH* 4.6 at temperatures from 277 to 333 K are shown in Figure 5. The majority of the hyperfine-shifted resonances show strong anti-Curie behavior (Figure 6), consistent with the existence of a thermal spin-state equilibrium (Iizuka & Morishima, 1974). Particularly noticeable are two broad resonances (M_a and M_b) which integrate to 3 protons. These exhibited very strong downfield shifts on increasing temperature, and M_a was observed to change the sign of its contact shift over the

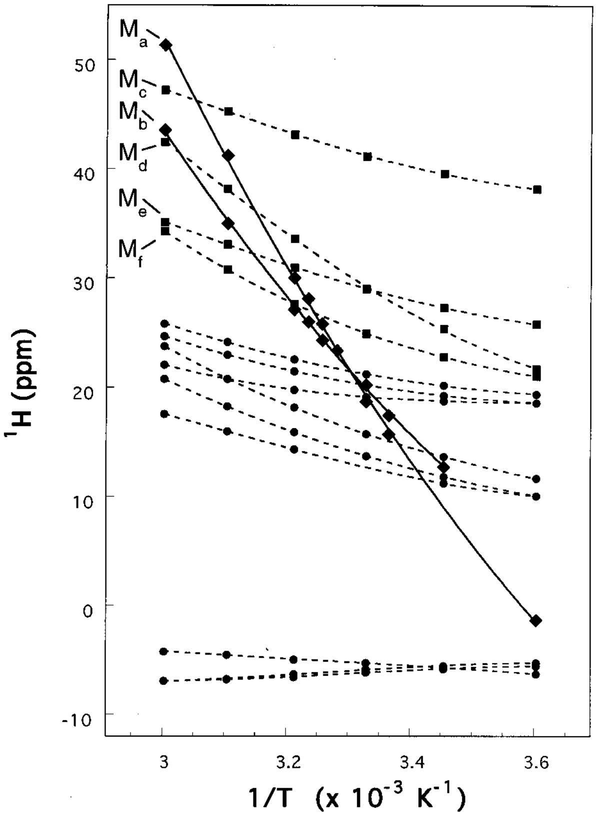

FIGURE 6: Curie plot of the chemical shift of the hyperfine-shifted resonances in the 500 MHz ^1H spectra (figure 5) of oxidized covR98C/H102M cytochrome b_{562} at pH* 4.5. (♦) Methyl resonanaces assigned to the methionine ligand ϵCH_3 protons (M_a and M_b in Figure 5); (■) methyl resonances assigned to the heme methyl groups (M_{c-f} in Figure 5); (●) hyperfine-coupled, single-proton resonances, currently unassigned.

temperature range studied. This behavior is very similar to that of the Met7 ligand methyl group in the wild type protein (Wu et al., 1991). Since no upfield-shifted three-proton resonances characteristic of the methyl groups of methionine ligands in low-spin ferricytochromes *c* (Wüthrich, 1969; Redfield & Gupta, 1971; Moore & Pettigrew, 1990) can be observed,[3] we suggest that M_a and M_b are the ϵCH_3 resonances of two axial methionine ligands. At 300 K, T_1 relaxation times for both these resonances are <1 ms.

The four heme methyl resonances, while presently not assigned to specific heme positions, can be identified (M_{c-f}, Figure 5), and the mean methyl chemical shift of ~37 ppm at 320 K is significantly higher than that of the wild type protein (28 ppm (Wu et al., 1991)) or the single mutant with a covalent linkage, R98C (~19 ppm, unpublished results). The T_1 relaxation times of the heme methyl resonances, M_{c-f}, were measured as 11, 12, 19, and 21 ms, respectively. An additional downfield shifted methyl resonance, M_g, emerges from the diamagnetic envelope above 308 K. A very broad, single proton resonance is observed as the most upfield-shifted signal (δ ~−16.5 ppm, inset Figure 5) only below 280 K. Comparison with the spectrum of the wild type protein again suggests that this resonance arises from a methylene proton of one of the methionine axial ligands and is the only other resonance identified so far which is thought to come from the axial ligand side chains. Six single proton resonances are observed between 12 and 24 ppm, two pairs of which show strong NOE's in NOESY and 1D NOE difference experiments and are probably the 6 and 7 α-methylene heme protons. NOESY experiments with mixing times ranging from 5 to 200 ms have revealed very few other crosspeaks involving the hyperfine-shifted resonances. 1D NOE difference spectra have so far provided little more information, and complete assignment of the heme and ligand protons has not so far been possible, particularly since we have not been able to assign any of the *meso* protons. This is in contrast to experiments using the wild type protein and the single mutant R98C in which dipolar contacts involving the fast relaxing protons have been more readily observed (unpublished results).

All the resonances broaden significantly above 320 K such that many of the signals are no longer resolved. A similar effect (data not shown) is observed by raising the sample pH toward the pK_a (7.1 at 293 K) of the low- to high-spin transition (Barker et al., 1996). Under the conditions used so far, neither the high-pH form of covR98C/H102M nor the oxidized single variant, H102M, at any pH* value gives rise to any hyperfine-shifted signals. We have varied the solution pH and the temperature of the experiments and also carried out experiments at lower field (300 MHz), but none of these have had any significant effect on the spectra. However, many protein signals within the normal diamagnetic envelope are still observable from these samples, and reduction leads to typical spectra of the diamagnetic, ferrous species. We suggest that the hyperfine-shifted resonances expected from these high-spin oxidized species are broadened beyond detection by chemical or spin exchange between species exhibiting very different hyperfine coupling properties.

DISCUSSION

NMR evidence clearly shows that both variant proteins, H102M and covR98C/H102M, have two methionines ligated to the heme iron in the reduced state between a solution pH of 4.5 and 8.5. Analysis of spectra of the single variant, H102M, is complicated by the observation of two sets of ligand resonances due to the presence of heme bound in two orientations, as described for the wild type protein (Wu et al., 1991; Barker et al., 1995). In addition, these spectra are pH dependent, and in particular, the resonances from the methionine ligand protons are quite sensitive to pH in the region of maximum stability of the heme/protein complex. The signals broaden significantly as the pH is taken below 6 or above 7.5. This may reflect a decrease in the stability of the complex due to an increased heme dissociation rate, and this has so far precluded detailed NOESY studies of either the low- or high-pH form of this protein.

With a covalent bond between heme and protein, the double variant, covR98C/H102M, is more amenable to structural study, although attempts to crystallize this protein have so far not been successful. However, results from NOESY spectra of the ferrous protein enable us to get some measure of the spatial arrangement of the methionine side chains with respect to the heme. Typical data are shown in Figure 4. We have used the NOE's observed to estimate distances between these methionine side chain protons and the heme protons, using the distance between geminal β or

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[3] This is in contrast to the spectrum of single mutant covR98C (unpublished results) in which a broad methyl resonance can be observed between 0 and −8 ppm at temperatures below 320 K.

γ protons as a reference. NOE's between the methionine ligand protons and the heme substituents can be used to define the conformation of the side chain and therefore the stereochemistry at the coordinating sulfur atom (Senn & Wüthrich, 1985; Santos & Turner, 1993; Costa et al., 1994).

Our analysis suggests that the side chain conformation of Met7 in the covalent R98C/H102M is very similar to that observed in the wild type protein and that the stereochemical configuration at the sulfur ligand is consistent with the *R* stereochemistry observed in the crystal structure of the wild type protein (Hamada et al., 1995). In particular, the Met7 methyl protons give strong crosspeaks with the β-*meso* proton and aromatic protons of Phe65, Phe61, and Tyr105. The Met7 methylene protons closest to the heme, γ' and β', contact the γ-*meso* and δ-*meso* protons, respectively, as observed in the wild type NOESY spectrum (major isomer (Barker et al., 1995)) and as expected from the crystal structure. Additionally, the chemical shifts of the Met7 protons in this double variant and the major isomer of the wild type protein are very similar. Furthermore, the chemical shifts and NOE patterns observed for the aromatic residues characterized here are also very similar to those observed in the major isomer of the wild type protein. Taken together, these observations suggest that, on the Met7 side of the heme, the structure of the protein and of the heme/protein interaction are little perturbed by either the covalent linkage or by the ligand substitution on the opposite side of the heme.

The analysis has also enabled us to model the Met102 side chain conformation. The Met102 methyl group (δ -3.15 ppm) gives strong NOE's with the δ-*meso* proton (δ 9.89 ppm) and the 1-methyl (δ 3.69 ppm), and a weak NOE to the α-*meso* proton (δ 9.50 ppm). This places the Met102 methyl group above pyrrole ring A on the α-*meso* side of the β,γ-*meso* axis. Both γ-methylene protons of the Met102 side chain (δ -4.27 and -2.56 ppm) give NOE's to the β-*meso* proton (δ 9.95 ppm), the former giving the stronger NOE. The β-methylene protons do not appear to give any NOE's to heme group protons. This suggests that the side chain of this ligand residue does not lay in an extended conformation across the heme as does the Met7 side chain in both the wild type protein and this double variant, but rather the β-methylene protons of Met102 must lie further above the heme plane than either of the γ-methylene protons. This is also suggested by the smaller heme ring-current shift experienced by both of the Met102 β-methylene protons. The signal (δ 2.56 ppm) assigned to the proton on the α-carbon of Met102 gives a strong NOE to the 5-methyl resonance (δ 3.82 ppm) and a weak one to the β-*meso* heme proton.

The resulting model for the structural relationship between the different protons is shown in Figure 7. In this figure, the structure of the Met7 side chain and the heme group is taken from the coordinates of the wild type protein (Hamada et al., 1995), while the Met102 side chain conformation is based upon the NOE constraints obtained in this work. We have used these qualitative NOE contacts to constrain a model of a "free" methionine residue ligated to the heme iron with an Fe−S bond length (2.34 Å) the same as that observed for the Met7 ligand in the wild type structure[4] (Figure 7). We suggest that this is a first approximation to the structure of the Met102 ligand in this covalent R98C/H102M protein. This analysis strongly suggests that the configuration at the Met102 sulfur atom is of *S* stereochemistry, which is therefore opposite to that observed for the Met7 sulfur ligand (*vide supra*). More significantly, for interpretation of the spectroscopic data (Barker et al., 1996), the orientations of the two methionine−sulfur lone pairs about the iron−sulfur axes predict a torsional angle between them of 110° (±10°). Interpretation of the spectroscopic data and proper comparison with the ligand structure in bacterioferritin will require knowledge of the structure of the ligand environment in the ferric state.

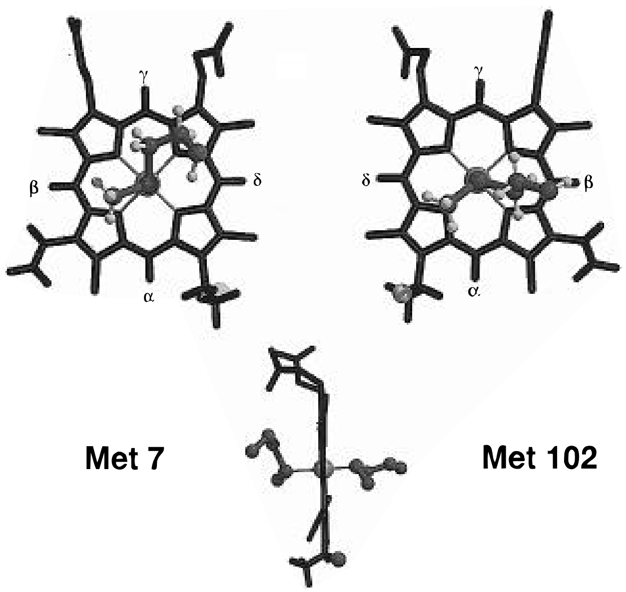

FIGURE 7: Conformation of the side chains and α-carbon of the methionine ligands, relative to the heme group, suggested by the NOE contacts described in the text. Top left: Met7, viewed down the S−Fe axis, as observed in the crystal structure of the wild type protein (Hamada et al., 1995). Top right: Met102, viewed down the S−Fe axis. Bottom: View down the $\delta-\beta$ *meso* heme axis. The coordinates for the heme group are taken from the structure of the wild type protein except that the 2-substituent has been modified to take account of the new thioether linkage at this position. The location of the thioether sulfur atom is indicated by the sphere attached to the 2-α-carbon. Figure drawn with Molscript (Kraulis, 1991) and rendered with Raster3D (Merritt & Murphy, 1994).

The conformation of the Met102 sidechain (Figure 7) predicted by these initial results requires that the polypeptide backbone at this position adopts a conformation slightly different to that observed in the wild type structure which has a histidine at 102. The side chain conformation (χ_1 and χ_2, $-70°$ and $+179°$, respectively) is one of the rotamers most commonly found in proteins (Dunbrack & Karplus, 1993), as is the conformation of the Met7 ligand ($-73°$, $-60°$). Following the identification of an unusual methionine ligand conformation in *Desulfovibrio vulgaris* cytochrome c_{553} (Blackledge et al., 1995b), it has been noted that the conformational freedom available to methionine may facilitate its use as a heme iron ligand when in a variety of polypeptide contexts (Blackledge et al., 1995a). We are currently trying to determine the full structure of the protein in both oxidation states by X-ray crystallography to facilitate

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[4] It is possible that, with two methionines ligated, the Met7 Fe−S bond may be longer, since Fe−S bond lengths of 2.64 and 2.69 Å are observed in the crystal structure of (ferric) bacterioferritin. These bonds are about 0.3 Å longer than observed in structures of His-Met ligated cytochromes *c* (Moore & Pettigrew, 1990). This might be indicative of a *trans* effect of methionine ligation although the effect could be oxidation state dependent.

a detailed analysis of the joint effects of the ligand substitution and the covalent linkage. It should be noted that this protein, covR98C/H102M, is the first *c*-type cytochrome to be described in which the ligand within the ubiquitous heme attachment motif (-CXYCH-) is not a histidine.[5] It will be interesting to ascertain whether or not a methionine ligand can be accommodated when both cysteine residues are present and attached to the heme.

An analysis by NMR of the single variant, H102M, similar to that described above has not so far been possible. The NOE patterns and side chain chemical shifts of the Met7 residue in this protein suggest significant difference in the conformation of this ligand when compared to the wild type and double variant. However, the comparison of spectra has been made under conditions in which the single mutant is a mixture of species which are in rapid exchange, that is, at a solution pH close to the pK_a for dependence of the chemical shift properties of the ligand protons on the solution pH. It cannot be excluded that there may be other conditions (low pH) in which the Met7 ligand protons have spectral properties similar to those observed in the wild type and double mutant proteins. The comparison at other pH values is hampered by the low affinity of the protein for heme at the extremities of the pH titration (Figure 2) and the variation with pH of the heme orientation ratio. The possible origins of the pH dependence of the properties of this protein have been discussed earlier (Barker et al., 1996), and as mentioned there, prime targets for the titratable group involved are the heme propionates or one of the side chains of the acidic residues, Glu4 and Glu8, which are hydrogen bonded to the heme propionates in the structure of the wild type holoprotein (Hamada et al., 1995).

Structural analysis of the heme and ligand residues of the oxidized covR98C/H102M protein in its low-spin form (i.e., at low pH) has not so far been possible. 2D NOESY and 1D NOE difference experiments (Banci et al., 1994) have not revealed any detectable NOE's involving the protons of M_a and M_b which we have assigned to the methionine ligand methyl groups. This is not surprising since nonselective T_1 measurements show that these protons relax very fast with T_1 values <1 ms. In addition, we have so far only been able to identify one other resonance from the methionine ligand methylene protons. Consequently, we have no information concerning the structure or stereochemistry at the methionine ligand side chains in the oxidized state. The low information content of the NOE experiments performed to date appears to be peculiar to this variant since control experiments with the wild type and single variant, R98C, allow the complete assignment of the heme group protons, and some of the ligand protons (data not shown). While significant progress has been made in the structure determination by NMR of both high- and low-spin paramagnetic proteins (Banci et al., 1995; Clark et al., 1996), homonuclear ^{1}H NMR techniques have only provided enough information for structure determination in a few ideal cases. The following properties of the covR98C/H102M variant of cytochrome b_{562} may preclude a definitive structural analysis by homonuclear ^{1}H NMR techniques.

At ambient temperatures, the low-pH form of covR98C/H102M appears to be a mixture of spin states in thermal equilibrium as shown by the temperature dependence of the ^{1}H NMR spectrum. This behavior has been observed for wild type cytochrome b_{562} but is in contrast to most other low-spin, six-coordinate cytochromes (Wüthrich, 1970; Wu et al., 1991). In the wild type protein, this equilibrium is suggested to involve low-spin ($S = {}^1/_2$) and high-spin ($S = {}^5/_2$) states, both six-coordinate. The covalent linkage in this protein does affect this spin equilibrium by stabilizing the low-spin state, since the single mutant, R98C, with the same thioether linkage between heme and protein, is almost entirely low-spin at all accessible temperatures and has a spectrum with a much weaker anti-Curie dependence on temperature (Barker and Freund, unpublished results). Using the analysis of Wu et al. (1991), the mean chemical shift of 37ppm for the heme methyl resonances in the ferric bis-methionine-coordinated protein described here suggests a 30% population of a high-spin state.

Of the spectroscopic data concerning this covalent, double mutant presented previously (Barker et al., 1996), only the EPR (at 10 K) has observable contributions from a high-spin component but this could be an artifact of freezing. The high-spin species in the covR98C/H102M protein at low pH, implicated by the temperature dependence of the NMR spectra, may be related to that observed in the wild type protein and remain 6-coordinate (Wu et al., 1991). However, there is an additional possibility that it is related to the high-spin species observed at high pH, since the pK_a for the process is lowered by increasing temperatures (Barker, unpublished results). The oxidized protein does not unfold until much higher temperatures under these conditions ($T_m = 87$ °C at pH 5; Barker and C. M. Johnson, unpublished results). At the highest temperature used for the NMR spectra in Figure 5, we estimate that the pK_a for the coordination-state change (Met-Met to Met-X) is 6.0. This predicts that, at this temperature, the concentration of the "high pH" high-spin species is 10% of the total, which cannot account for the larger proportion of high-spin material suggested by the NMR results. There is clearly the possibility of more than two species being involved in the spin state equilibria, one or both of which may be partially unfolded proteins (Clark et al., 1996).

We have been unable to observe any hyperfine-shifted resonances from the high-spin, "Met-X" coordinated states of either of the ferric proteins, that is, the H102M protein at any pH or the covR98C/H102M protein above pH 7.1. We currently only have one explanation for this which is that, in these states, the proteins exist as two or more species having significantly different hyperfine contact shifts, interconverting at a rate sufficient to broaden all the paramagnetically shifted resonances. The simplest scenario one could imagine which could give rise to this effect is an interconversion between Met-Met and Met-X coordinated states, where Met-X is possibly a 5-coordinate state as previously discussed (Barker et al., preceding paper). In other words, the "labile" methionine ligand is coming on and off, with the equilibrium lying well to the Met-X ("off") side, since at room temperature only the high-spin state is observed in the optical and MCD spectra. In the H102M protein, one could argue that the heme orientation equilibrium might also contribute to this line broadening. The low affinity of this protein for heme and high heme reorientation rate (data not

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[5] It has been suggested (Berks et al., 1995) that a -CXYCK- sequence derived from the gene for the "hexaheme" cytochrome *c* nitrite reductase of *E. coli* (Darwin et al., 1993) could be a heme binding site with the lysine providing a ligand. To date, no other evidence for this has been obtained.

shown), even in the reduced state, suggest a higher heme dissociation rate than has been observed in the wild type protein and other mutants of this cytochrome (Barker, unpublished results). However, even in this destabilized mutant, the rates do not appear fast enough to result in the chemical exchange phenomenon observed. Clearly, this mechanism cannot be the cause of the line broadening in the double mutant with covalently attached heme.

While the covR98C/H102M protein may be an interesting candidate to test methods of structural determination of paramagnetic proteins by NMR (Banci et al., 1994), the existence of these multiple equilibria between different spin states may provide alternative relaxation pathways resulting in loss of correlation in our homonuclear NMR experiments. Heteronuclear methods (Muskett et al., 1996) combined with calculation of distances from hyperfine shift parameters as well as NOEs (Banci et al., 1995) will probably be required to provide enough information for a detailed structure calculation of this type of paramagnetic protein. Although we are continuing experiments to assign the ^{1}H NMR spectrum of the ferric from of covR98C/H102M, the analysis may be easier if we can isolate a protein in which the bis-methionine ligation state is stabilized. This could be achieved by removing the titratable group involved in the pH-linked spin-state transition so that the protein is locked into its low-pH state. We hope that such a protein in its paramagnetic state will be more amenable to accurate distance estimation by measurement of NOE's.

CONCLUSIONS

The novel bis-methionine-ligated cytochromes described here, particularly the *c*-type R98C/H102M, have unprecedented electronic and functional properties. Our NMR results suggest that the ligand structure is also unprecedented. In these cases, the energetics of ligation to the heme iron is in a delicate balance with the stability of the protein fold. The well-known, unusual affinity of ferrous iron for the thioether ligands (Margoliash & Schejter, 1966; Schejter & Plotkin, 1988; Schejter et al., 1991; Barker & Mauk, 1992) dominates the protein structure in this oxidation state, and given a flexible backbone such as the cytochrome b_{562} C-terminal helix, ligand conformations which may be otherwise energetically suboptimal can be accommodated. In the ferric state, methionine ligation to the heme is obviously weaker, and in the cases described here the heme/protein interaction depends on the energetics of the polypeptide structure, including the ionization of individual groups. If certain conformations and ligation states are that close in energy, it would not be surprising that they should interconvert resulting in a dynamic system.

In such a system, one would predict that the provision of ligands which have a higher affinity for ferric iron than does the methionine thioether could replace the methionine as a ligand in this oxidation state. There is a well described "natural" precedent for this. Mitochondrial cytochrome *c*, and variations of it, have now been shown to undergo a variety of oxidation state-dependent ligand rearrangements (Barker & Mauk, 1992; Ferrer et al., 1993; Hawkins et al., 1994; Theodorakis et al., 1995; Schejter et al., 1996). We have designed and isolated suitable multiple mutants of these H102M containing cytochromes b_{562}, and indeed many of these can be observed with 6-coordinate ligation states but with different ligand combinations in each oxidation state (Barker, Nerou, Cheesman, and Thompson, manuscript in preparation).

ACKNOWLEDGMENT

We thank Dr. David Neuhaus (MRC Laboratory of Molecular Biology) for access to the DRX 300 instrument.

SUPPORTING INFORMATION AVAILABLE

Proton chemical shifts of the heme group and selected amino acid residues for the ferrous H102M and R98C/H102M proteins (two tables); list of NOE contacts used to build the model for the Met102 ligand conformation (one table); 1D reference traces of the three ferrocytochromes b_{562} (WT, H102M, and R98C/H102M) at 27 °C (one figure) (6 pages). Ordering information is given on any current masthead page.

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BI961128P