# Bis-Methionine Ligation to Heme Iron in Mutants of Cytochrome $b_{562}$. 1. Spectroscopic and Electrochemical Characterization of the Electronic Properties[†]

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*Received May 13, 1996; Revised Manuscript Received August 19, 1996*[⊗]

ABSTRACT: We have generated mutants of cytochrome $b_{562}$ in which the histidine ligand to the heme iron (His102) has been replaced by a methionine. The resulting proteins can have bis-methionine coordination to the heme iron, but the stability of this arrangement is dependent on oxidation state and solution pH. We have used optical, MCD, and EPR spectroscopies to study the nature of the heme coordination environment under a variety of conditions. Optical spectra of the reduced state of the single variant, H102M, are consistent with bis-methionine ligation. In its oxidized state, this protein is high-spin under all conditions studied, and the spectroscopic properties are consistent with only one of the methionine ligands being coordinated. We cannot identify what, if anything, provides the other axial ligand. A double variant, R98C/H102M (in which the heme is covalently attached to the protein through a *c*-type thioether linkage), is also bis-methionine coordinated in the ferrous state, but has significantly different properties in the oxidized state. With a p$K_a$ of 7.1 at 20 °C, the protein converts from a low-spin, 6-coordinate heme protein at low pH, to a high-spin species, similar to the high-spin species observed for the single variant. Our spectroscopic data prove that the low-spin species is bis-methionine coordinated. The reduction potential of this bis-methionine species has been measured using direct electrochemical techniques and is +440 mV at pH 4.8. The electrochemistry of these proteins is complicated by coupled coordination-state changes. Proof that the ferrous state is bis-methionine coordinated is provided by NMR results presented in the following paper.

The role of the axial ligands to iron in modulating the properties and reactivity of the heme prosthetic group within a given protein structure has been of considerable interest for some time (Wuttke & Gray, 1993). Low-spin, bis-coordinate heme centers in proteins typically have a role in rapid electron transfer reactions since redox changes at the metal ion lead to little structural rearrangement. Histidine, lysine, methionine, and cysteine are the only amino acids with side chains that have ligand field strengths sufficient to generate the low-spin state of the heme although the terminal α-amino group of the polypeptide must now be added to this list since its observation as a ligand in cytochrome *f* (Martinez et al., 1994). Of the possible pairwise combinations of these ligands, only five have been observed in low-spin cytochromes. Bis-histidine and methionine−histidine coordination are by far the most commonly observed ligation combinations in these cytochromes (Moore & Pettigrew, 1990). Lysine−histidine coordination has been observed in the alkaline form of mitochondrial cytochromes *c* (Ferrer et al., 1993), while cytochrome *f* is currently the only example of α-amino−histidine ligation. Bis-methionine ligation has only recently been observed in bacterioferritin, as isolated from a variety of sources (Cheesman et al., 1990, 1992, 1993; George et al., 1993).

A number of studies have now reported the alteration, by semisynthesis or site-directed mutagenesis, of the axial ligands within a variety of 6-coordinate heme proteins (Raphael & Gray, 1989, 1991; Mus-Veteau et al., 1992; Wallace & Clark-Lewis, 1992; Dolla et al., 1994; Ubbink et al., 1994). None have generated a bis-methionine coordinated cytochrome for study of the properties of this system. There is also increasing interest in cytochromes which undergo oxidation state ligand rearrangements (Barker & Mauk, 1992; Ferrer et al., 1993; Hawkins et al., 1994; Theodorakis et al., 1995; Schejter et al., 1996). We are using the 4-α-helical bundle cytochrome $b_{562}$ as a scaffold for the synthesis of cytochromes with novel oxidation state-linked conformational properties. This protein, from the periplasm of *Escherichia coli* (Itagaki & Hager, 1966; Hamada et al., 1995), is a low-spin, methionyl−histidinyl coordinated cytochrome with noncovalently bound heme (Bullock & Meyer, 1978; Meyer & Bullock, 1978; Moore et al., 1985;

[†] This work was supported by ZENECA Group plc, the Medical Research Council, and the DTI under a Protein Engineering LINK program (P.D.B.), and also by the Biomolecular Sciences Committee of the BBSRC and EPSRC via the UEA Metalloprotein Centre (M.R.C., A.J.T.). P.d.O. was supported by the Calouste Gulbenkian Foundation, Lisbon.

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

S0006-2960(96)01127-0 CCC: $12.00 

Wu et al., 1991). We report here the generation of a bis-methionine coordinated cytochrome $b_{562}$. We are also able to generate cytochrome $b_{562}$ proteins which have heme covalently attached to the protein through *c*-type, thioether linkages (Barker et al., 1995). Therefore, we can compare two cytochrome $b_{562}$ proteins with bis-methionine ligation with and without covalently attached heme. The proteins described here exhibit both oxidation state-dependent and pH-dependent spectral properties consistent with changes in coordination state. We have used EPR and MCD spectroscopies to characterize the ferric states of these proteins. We have studied the direct electrochemistry of these proteins using cyclic voltammetry and found that the electrochemical response is complicated by coupled reactions in homogeneous solution. Using direct electrochemistry, we have been able to identify the reduction potential of a bis-methionine coordinated protein across the pH range studied, even under conditions in which it is not the most stable species. In the following paper (Barker & Freund, 1996) NMR analyses of both oxidation states are presented, which have provided information concerning the structure of the methionine ligands and the heme pocket in the reduced state. The electronic and structural consequences of this coordination state are discussed and comparisons made with the known structure and properties of bacterioferritin.

## MATERIALS AND METHODS

*Cloning, Mutagenesis, and Protein Purification.* The cloned gene of cytochrome $b_{562}$ was mutated using the PCR protocol described before (Barker et al., 1995), with the reverse primers containing suitable codons for constructing the H102M and R98C/H102M mutant genes. Growth of *Escherichia coli* (Strain NM554) and protein purification were also as previously described. H102M was isolated only as the apoprotein since the small amount of holoprotein observed in cells and cell extracts did not survive the purification due to heme removal by chromatography resins. Holoprotein was generated by addition of bovine hemin (Sigma) in 0.1 M NaOH followed by re-equilibration at the desired solution pH. R98C/H102M was isolated as the apoprotein and also as a holoprotein, with a covalently-linked heme. These were the only two cytochrome $b_{562}$ species observed following expression of this mutant. All chromatography was carried out using a Waters 625 system equipped with a 996 photodiode array detector. Optical spectra were recorded on a Cary 3E spectrophotometer. The pH dependence of the optical spectra was determined by equilibration of samples of identical protein concentration in buffers of differing solution pH containing 0.1 M potassium chloride. Buffers used were MES (Sigma), triethanolamine, and diethanolamine (BDH). Molecular masses were measured by electrospray ionization mass spectrometry under conditions which dissociate heme from the wild-type protein as previously described (Barker et al., 1995).

*MCD and EPR Spectroscopy.* All protein samples were in $D_2O$-based buffers (either CHES or HEPES, Sigma) containing potassium chloride. Deuterium oxide was from Fluorochem. pH* is the apparent pH of these buffers measured with a standard glass pH electrode. Electronic absorption spectra were recorded on a Hitachi U4001 spectrophotometer. EPR spectra were recorded on an X-band ER-200D spectrometer (Bruker Spectrospin) interfaced to an ESP1600 computer and fitted with a liquid helium flow cryostat (ESR-9; Oxford Instruments). Magnetic circular dichroism (MCD) spectra were recorded on either a circular dichrograph, JASCO J-500D, for the wavelength range 280−1000 nm, or a laboratory-built dichrograph for the range 800−2500 nm (Gadsby & Thomson, 1990). MCD spectral intensities were linear with magnetic field at room temperature and are expressed per unit magnetic field, as $\Delta\epsilon$ ($M^{-1}$ $cm^{-1}$ $T^{-1}$).

*Protein Electrochemistry.* The details of the electrochemical apparatus, cyclic voltammetric experiments, and electrode preparation are described elsewhere (Barker et al., 1996). Direct electrochemistry of the wild-type protein and variants was achieved at either a gold electrode modified with the hexapeptide, KCTCCA, or at an edge−plane graphite electrode with neomycin added to the solution (1 mM) as a promoter. The reduction potentials measured were independent of the surface used. Protein was exchanged into a mixed buffer system described previously (Barker & Mauk, 1992). Due to the low affinity of the H102M variant, the electrochemistry of this holoprotein was studied in the presence of at least 2-fold excess of the apoprotein. In addition, the protein was reduced prior to study and the reductant removed on a desalting column equilibrated in the mixed buffer. Studies of the reduced protein required anaerobic conditions and hence were carried out in a glovebox. The same was true of the reduced covR98C/H102M protein at high pH, which also readily autoxidizes. Potential measurements were made against a saturated calomel electrode (Radiometer) and converted to the SHE scale.

## RESULTS

*Characterization of H102M Cytochrome $b_{562}$.* The optical spectra of oxidized and reduced H102M apoprotein freshly reconstituted with hemin are shown in Figure 1A. The spectrum of the ferric protein shows no similarity to the spectrum of the oxidized wild-type protein but has characteristics, particularly the high-spin marker band at 610 nm, indicative of a high-spin ferric iron containing system. The spectrum of the holoprotein in the ferric state is independent of pH between values of 4.0 and 9.5, although the affinity of the protein for oxidized heme is significantly reduced below pH 5 and above pH 8.5 (Barker and Nerou, unpublished results).

The optical spectrum of the reduced protein (Figure 1A) is typical of a six-coordinate cytochrome although the wavelength maxima of the Soret and visible bands (430, 532, and 561.5 nm at pH 5) are significantly red shifted from those of the reduced wild-type protein (427, 530, and 561.5 nm). The optical spectrum of this reduced protein is pH dependent (Figure 1B). The Soret and α-band maxima shift slightly further to the red (432 and 563.5 nm, respectively) with increasing pH. Figure 1C plots the wavelength of the Soret maximum as a function of pH. The dependence of the extinction coefficient upon pH is small; the largest change is at 432 nm which is plotted in Figure 1D. These data have been fitted to an equation, describing the lines in Figures 1C and 1D, in which each property is a function of two noninteracting p$K_a$s, which gives significantly better correlation than a function describing just one p$K_a$. The fit to the wavelength data gives p$K_a$ values of 6.2 ± 0.2 and 7.6 ± 0.8; the fit to the absorbance data gives p$K_a$ values of 6.1

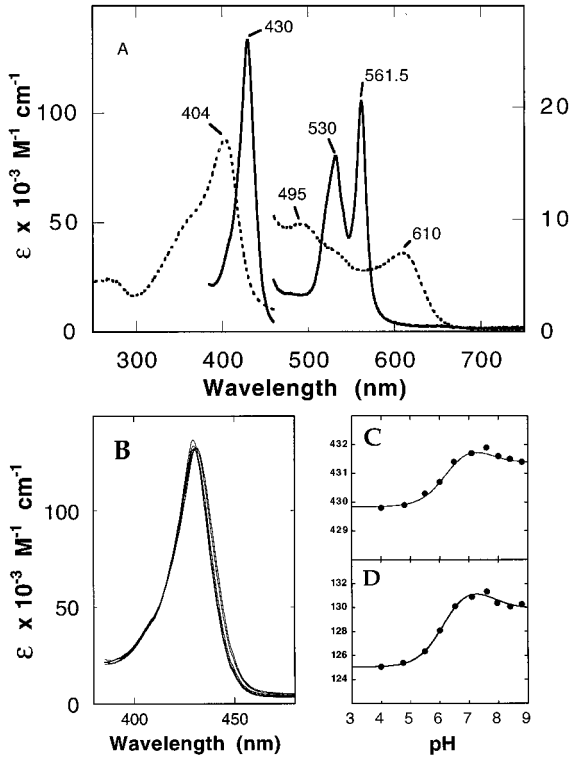



FIGURE 1: Optical spectra of H102M cytochrome $b_{562}$. (For buffer conditions, see text.) (A) Oxidized (dotted) and reduced (solid) protein at pH 5.0; (B) pH dependence of the Soret absorption of the reduced protein between pH 4.1 and 8.8. (C and D) Dependence on pH of the wavelength maximum (C) and extinction coefficient at the apex (D) of the Soret band of reduced protein. The curves in (C) and (D) describe the best least-squares fit of each property to a titration function involving two independent titratable groups.

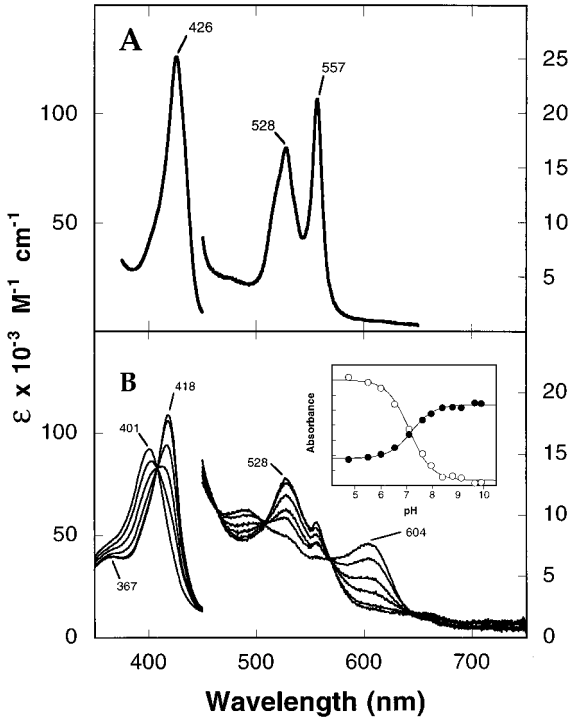



FIGURE 2: Optical spectra of covR98C/H102M cytochrome $b_{562}$. (For buffer conditions, see text.) (A) Reduced protein at pH 7.1. (B) Oxidized protein at pH values 4.8, 6.1, 7.1, 7.8, 8.5, and 9.5. Inset: Dependence of the absorption at 418 nm (open circles) and 401 nm (closed circles) on pH. The curves describe the best fit to a function involving one titratable group.

$\pm$ 0.1 and 7.7 $\pm$ 0.5. Clearly there is insufficient data to describe the higher p$K_a$ transition, but we believe the effect to be real and not due to a change in heme affinity under the conditions of the experiment.

*Characterization of Covalently-Linked R98C/H102M Cytochrome $b_{562}$.* Following expression of this mutant in *E coli*, approximately 150 mg of apoprotein was purified, from the cell periplasm and culture medium, per liter of culture. Small amounts of a single holoprotein were detected in the same culture fractions by ion exchange chromatographic analysis, and this species was purified to yield about 0.5 mg/L of culture. The following analytical results suggested that this protein had heme covalently attached to the polypeptide through the cysteine residue at position 98 (Barker et al., 1995). The molecular mass, measured under conditions which dissociate heme from noncovalently bound cytochrome, was 12 338 Da, which corresponds to the theoretical mass of apoprotein plus heme. The purified protein stained for heme following SDS−PAGE analysis. The protein did not react with 4,4′-dithiodipyridine and gave no reaction with maleimide containing reagents. The heme could not be extracted by treatment with acidified butanone. The reduced pyridine hemochrome spectrum had absorption maxima (e.g., an α-band at 553nm) consistent with the loss of one heme vinyl group (Barker et al., 1993, 1995). Proof of the existence of a single *c*-type thioether linkage was provided by NMR spectroscopy (Barker & Freund, 1996).

The optical spectrum (Figure 2A) of this covalently bound holoprotein (covR98C/H102M) in the reduced state is blue shifted relative to that of the noncovalent single variant, H102M, and is not dependent on the solution pH between values of 4.5 and 9.0. However, the spectrum of the oxidized cytochrome is pH dependent, appearing qualitatively like that of the noncovalent, H102M protein at high pH. The Soret and high-spin marker bands are blue-shifted, relative to the H102M protein, at 401 and 604 nm, respectively. At low pH, the spectrum appears characteristic of a low-spin cytochrome. An example of a spectral titration is shown in Figure 2B. The absorbance changes at 404 and 418 nm are plotted in Figure 2B inset and fitted to an equation describing the change in absorbance with the titration of a single ionizable group. The mean p$K_a$ value determined from these and other wavelengths is 7.10 ($\pm$0.05) at 25 °C. The spectrum of the oxidized protein at low pH also shows weak, broad bands at around 700 nm (Figure 3A), characteristic of iron to methionine sulfur charge transfer bands, which are not present at high pH.

*MCD Characterization of the Ferric Proteins.* The room temperature electronic absorption and MCD spectra of the covR98C/H102M variant at pH* 4.8 and 9.2 and of the H102M cytochrome $b_{562}$ variant at pH* 7.0 are shown in Figures 3, 4, and 5, respectively. The initial assignments of the spin states observed in these three species from the optical spectra are supported by the MCD spectra of these three species shown in Figures 3B, 4B, and 5B. CovR98C/H102M at pH* 4.8 gives rise to a dispersion-shaped Soret feature (Figure 3B) more than an order of magnitude more intense than the Soret bands of covR98C/H102M at pH* 9.2 and H102M (Figures 4B and 5B, respectively). The spectrum in Figure 3B is consistent with a low-spin cytochrome. High-spin ferric marker bands are readily observed in the MCD spectrum where they appear as a dispersion-shaped features (Brill & Williams, 1961). Both the intensity and the shape

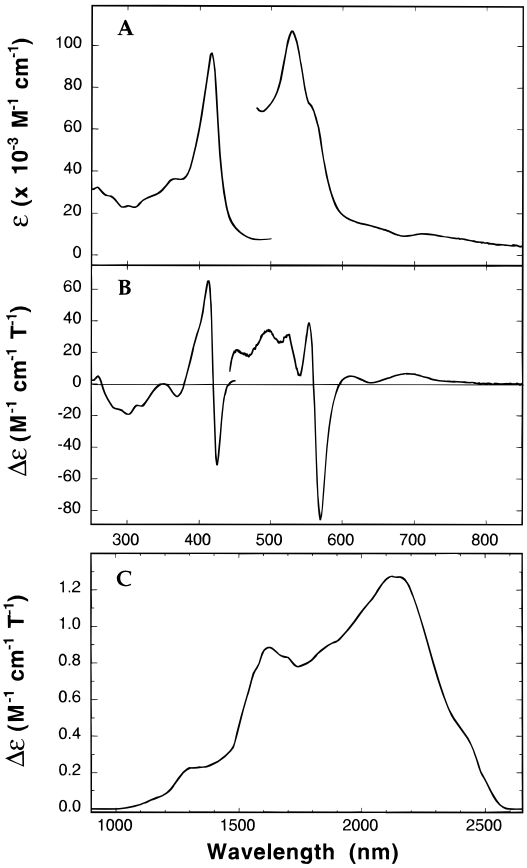



FIGURE 3: Optical (A), UV/visible MCD (B), and NIR-MCD (C) spectra of oxidized covR98C/H102M cytochrome $b_{562}$ (80 or 880 $\mu$M) at pH* 4.8 (25 mM potassium acetate, 100 mM potassium chloride in $D_2O$).

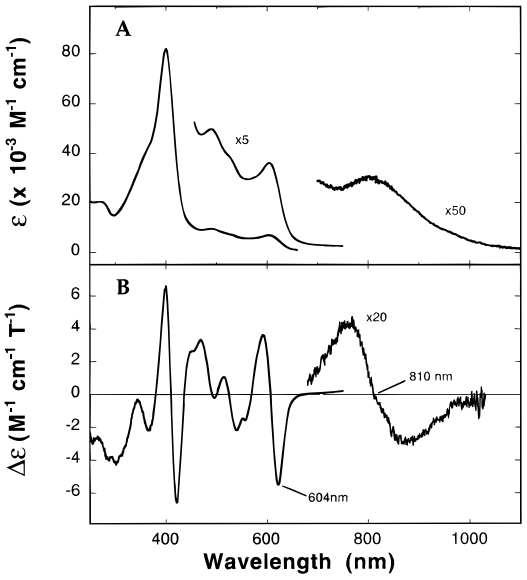



FIGURE 4: Optical (A) and UV/visible MCD (B) spectra of oxidized covR98C/H102M cytochrome $b_{562}$ (85 or 745 $\mu$M) at pH* 9.2 (25 mM CHES, 100 mM potassium chloride in $D_2O$).

of the MCD features corresponding to the absorption bands at 604 and 610 nm for covR98C/H102M at pH* 9.2 and H102M, respectively (Figures 4B and 5B), confirm that these features are high-spin charge-transfer transitions.

Ferric hemes in both spin states give rise to an informative charge-transfer band (CT) at longer wavelengths. In the low-spin case, this is a porphyrin($\pi$) $\rightarrow$ Fe(III) (d) ($a_{1u} \rightarrow e_g$) CT

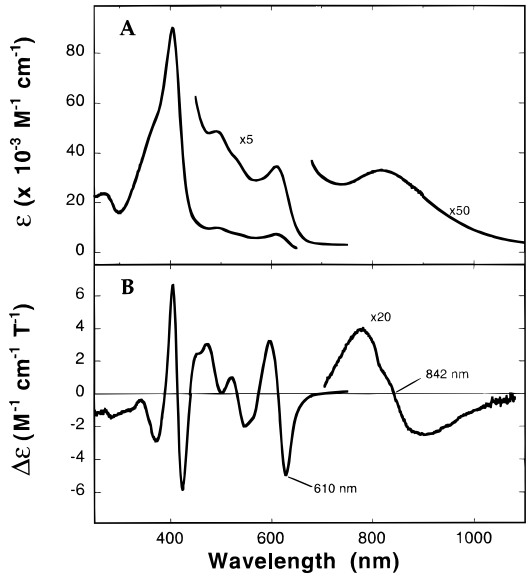



FIGURE 5: Optical (A) and UV/visible MCD (B) spectra of oxidized H102M cytochrome $b_{562}$ (156, 954, or 4460 $\mu$M) at pH* 7.1 (25 mM HEPES, 100 mM potassium chloride in $D_2O$).

transition (the NIR-CT band) found between 800 and 2500 nm. The precise energy is diagnostic of the axial heme ligands (Gadsby & Thomson, 1990; Cheesman et al., 1991). The room temperature NIR-MCD spectrum of covR98C/H102M at pH* 4.8 is shown in Figure 3C. To date, the only proteins shown to contain bis-methionine ligated ferric hemes are the bacterial ferritins which have a NIR-CT band near 2270 nm, the longest wavelength so far observed for this transition (Cheesman et al., 1990). The analogous band for covR98C/H102M at pH* 4.8 is seen at a similar wavelength ($\sim$2150 nm), confirming that this species has bis-methionine ligation. The weaker higher energy feature ($\sim$1600 nm) is always observed for this transition in naturally occurring low-spin ferric hemes and is believed to be a vibrational sideband (McKnight et al., 1991).

The presence of methionine ligands to low-spin ferric heme always gives rise to extra CT intensity between 600 and 700 nm, and such bands are observed in the low-spin spectrum of covR98C/H102M (Figure 3B) and are similar to those observed for the bacterioferritins (Cheesman et al., 1992). For the methionine/histidine liganded hemes of cytochromes *c*, this intensity is well recognized and is referred to as the "695 band" (Moore & Pettigrew, 1990). The NIR-CT band of high-spin ferric hemes is a dispersion-shaped feature which has also been attributed to a porphyrin $\rightarrow$ Fe(III) charge-transfer process (Cheesman et al., 1991). The NIR-CT bands observed from the high-spin species of covR98C/H102M at pH* 9.2 and H102M (Figures 4B and 5B) are centered at 810 and 842 nm, respectively.

*EPR Spectroscopy of the Ferric Proteins.* The X-band EPR spectra of the three main species described here at 10 K are shown in Figure 6. H102M at pH* 7.0 and covR98C/H102M at pH* 9.2 are clearly high-spin (Figure 6A,B) but both show twofold heterogeneity. The major component of the spectrum (Figure 6C) of the covR98C/H102M, at pH* 4.8, is the very broad low-spin feature ($g$ values 3.19 and 2.25). The spectrum has a sharp, high-spin ($g = 5.9$) component, but this accounts for $<5\%$ of the spins present in this sample and may be an artifact of freezing. The $g_z$ value (3.19) observed in the low-spin species is significantly

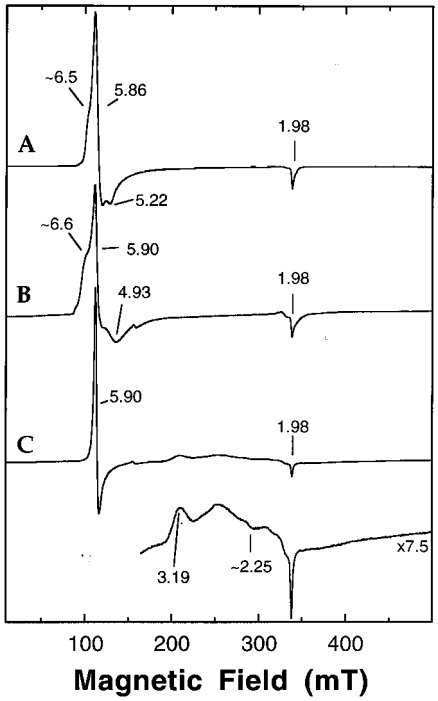



FIGURE 6: X-band EPR spectra at 10 K of (A) H102M cytochrome $b_{562}$ at pH* 7.1; (B) covR98C/H102M cytochrome $b_{562}$ at pH* 9.2; (C) covR98C/H102M cytochrome $b_{562}$ at pH* 4.8. The proteins were oxidized and the buffer conditions as given in figures 3−5. Spectra recorded at a microwave power of 2 mW and modulation amplitude of 1 mT.

higher than that observed for the bacterioferritins (∼2.88) (Cheesman et al., 1992, 1993), suggesting that the effective symmetry at the Fe(III) ion in this cytochrome $b_{562}$ derivative is higher than in the ferritins (see Discussion).

*Electrochemistry of the Bis-Methionine Coordinated Species.* Our spectroscopic studies clearly show that these proteins can exist in a variety of liganded forms in equilibria. Therefore, multiple, interconverting species might be expected to be detected electrochemically. Only dynamic electrochemical techniques, such as cyclic voltammetry, allow the electrochemical properties of the individual species to be observed. This requires direct electron transfer between the cytochrome $b_{562}$ proteins and electrodes. This has been achieved at a variety of electrode surfaces, the details of which are published elsewhere (Barker et al., 1996).

We describe, first, the results from electrochemistry of the covalently-linked, double variant, R98C/H102M, since there are conditions under which the electron transfer events are not complicated by changes in coordination state, that is, at low solution pH. At pH 4.85, with either oxidized or reduced protein, a perfectly reversible response was observed with a midpoint potential of +440 mV (±5 mV vs SHE). The peak currents were proportional to the protein concentration and to the square root of the scan rate, and anodic (oxidation) and cathodic (reduction) currents were always in a ratio of approximately 1 (Figure 7A). These data are consistent with the presence of a single electroactive species. As the pH of the solution was increased through the p$K_a$ of the spectral transition for the oxidized protein, the electrochemistry of this species became asymmetrical. The peak potentials and peak currents were very scan rate dependent and also dependent upon the oxidation state of the protein in bulk solution. The dependence of the ratio of the cathodic ($i_{pc}$) to anodic ($i_{pa}$) currents upon these parameters is shown in Figure 7A.

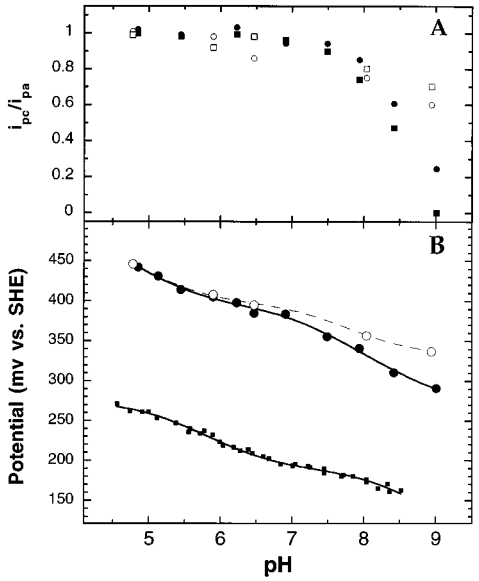
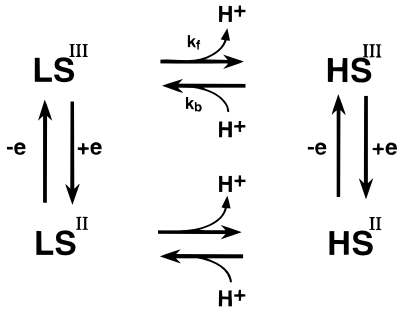



FIGURE 7: (A) Electrochemistry of covR98C/H102M cytochrome $b_{562}$. Ratio of cathodic ($i_{pc}$) to anodic ($i_{pa}$) currents from cyclic voltammograms at sweep rates of 1 mV/s (circles) and 200 mV/s (squares) as a function of pH. (B) Reduction potentials (mV vs SHE) of covR98C/H102M (circles) and wild type (squares) cytochrome $b_{562}$ as a function of pH. The protein was present in the bulk solution as either the ferricytochrome (closed symbols) or the ferrocytochrome (open symbols).

Scheme 1




Despite scanning over a wide potential range (down to −700 mV vs SHE), we did not observe an alternative electrochemical response, even under conditions in which 99% of the protein should be present as the oxidized, high-spin species (pH 9.1). Scheme 1 describes the equilibria affecting the electrochemistry, and the resulting thermodynamic square is similar to that of other conformationally active electrochemical systems (Barker & Mauk, 1992; Bond, 1994). Under conditions where the high-spin species ($HS^{III}$) is the most stable form of the ferricytochrome (above pH 7.1), the high potential species is the only one detected electrochemically. The electrochemical behavior is consistent with the rapid equilibration of this high-spin species ($HS^{III}$) with its low-spin counterpart ($LS^{III}$) preceding the reduction of this latter species by the electrode. When the protein is reduced in bulk solution ($LS^{II}$ is the only species present), the behavior is now consistent with the rapid equilibration of high- and low-spin species following the oxidation of the protein by the electrode.

The effect of this kinetically-coupled, electrochemical behavior on the measurement of the reduction potential of the protein is complicated and is considered in detail

elsewhere (Barker et al., 1996) where the rates of interconversion between the two species are also estimated. Figure 7B shows the pH dependence of the midpoint potential at low sweep rate (1 mV/s) when the bulk solution contains oxidized protein, together with the same potential measured with the protein in bulk solution reduced and at a higher sweep rate (200 mV/s). Also shown for comparison is the pH dependence of the midpoint potential of the wild-type protein.[1]

For the single variant, H102M, we expect to observe electrochemical responses complicated by the same type of kinetic scheme with the protein in either oxidation state and at all accessible pH values. The electrochemistry of this protein is complicated by the removal of heme from the protein by the electrode surface, even when the protein is reduced and an excess of apoprotein is present. Consequently, fewer data are available from this protein, but we have measured the midpoint potential of the electrochemical wave as +420, +300, and +240 mV (vs SHE at 20 mV/s) at pH 5.3, 6.2, and 7.0, respectively, with the protein in the reduced state. With the protein in either oxidation state, the current ratio varies only slightly with sweep rate and is never close to unity.

## DISCUSSION

We have isolated two types of cytochrome $b_{562}$ variant with methionine at residue 102 replacing the histidine found as a ligand to the heme iron in the wild-type protein. One has noncovalently bound heme, and in the other, heme is covalently attached to the protein. For the latter, double variant (covR98C/H102M), the NMR evidence presented in the following paper (Barker & Freund, 1996) clearly shows that the heme is attached through a single *c*-type thioether bond formed between the cysteine 98 thiol and the heme 2-α-vinyl carbon, as described previously for the single variant, R98C (Barker et al., 1995). This double variant is the first *c*-type cytochrome to be described with bis-methionine ligation to the heme iron and the first proven to have a ligand other than histidine in the heme binding motif, -CXYCH-. The mechanism of heme attachment to this protein will be the subject of a future publication (Barker and Nerou, unpublished results) and will not be considered further here. Both proteins studied here have unprecedented electronic and MCD spectra not previously observed from any cytochrome. Our analyses have revealed that their coordination states are sensitive to conditions of pH or oxidation state. Two distinct types of ligation to heme iron can be described and evidence for them is discussed here.

*Bis-Methionine Ligation.* In the reduced states of both proteins studied here (Figures 1 and 2) the ligands to the heme iron are provided exclusively by methionine (Barker & Freund, 1996). In their oxidized forms, the single and double variants clearly differ in the stability of this bis-methionine ligation state. Of the oxidized species described here, only the low pH form of the covalent, double variant is clearly low-spin, and all the spectroscopic evidence suggests that both methionines are ligated in this species. The NIR-MCD charge transfer band at 2150 nm (Figure 3C) is particularly diagnostic.

The features in the EPR spectrum of Figure 6C are unprecedented. Features at lower *g* values were not detected, but using $g_z = 3.19$ and $g_y \sim 2.25$ predicts that $g_x \sim 0.87$ on the basis that the sum of the squared *g* values is 16 for low-spin ferric hemes. Analysis of these values using the method of Taylor (1977) yields $\Delta/\lambda = 1.91$ and $V/\Delta = 1.13$ for the tetragonality and rhombicity parameters, respectively. Without the NIR-MCD data, these two parameters do not unambiguously identify the axial ligands, but the values calculated here have not been observed for other low-spin ferric hemes (Blumberg & Peisach, 1971). They are distinct from those calculated from the EPR spectra of the bis-methionine liganded heme in bacterioferritins: $\Delta/\lambda \sim 2.7$ and $V/\Delta \sim 1.9$. We attribute this difference to the orientations of the respective methionine ligands. In bacterioferritin, the twofold symmetry of the heme binding site (Frolow et al., 1994) ensures that the two lone pairs of each methionine sulfur lie in the same plane perpendicular to the heme. This leads to the high rhombicity values and is a situation analogous to that found for bis-histidine ligated hemes for which the ligand planes are parallel. As such histidine ligands are rotated toward the perpendicular arrangement, the $\pi$-interactions with the ferric ion then tend toward being equivalent in the *x*- and *y*-directions. This reduces the rhombicity leading to higher $g_z$ values and more intense NIR-MCD. A similar situation occurs here for the bis-methionine-liganded heme of mutant cytochrome $b_{562}$. The methionine ligands are effectively rotated from the "parallel" arrangement, resulting both in a $g_z$ value higher than those found for bacterioferritin and also in a high intensity NIR-MCD. The model for the Met102 side chain conformation we predict from NMR data in the following paper fits this analysis well, in that it predicts a torsional angle of 110° (±10°) between the lone pairs in the *ferrous* protein. If this angle is maintained in the *ferric* state, then the ligand field symmetry approaches the purely axial limit for which this angle would be 90° and is significantly different from the 180° arrangement observed in bacterioferritin which is close to a purely rhombic system.[2]

*Met-X Ligation.* In the single variant, with a noncovalently bound heme the ferric ion is high-spin, as identified by the 610 nm band observed in the MCD spectra, making it extremely unlikely that both methionines are bound to the heme iron. From the similarity of the MCD spectra, it appears that the heme ligation is the same in the high-pH form of the covalently attached, double variant. The exact nature of the "630" feature observed in the MCD spectra of

[1] The reduction potentials of the wild type protein above pH 8 in Figure 7 are higher than those previously published (Moore et al., 1985). This is due to the fact that the oxidized, wild type protein undergoes a deprotonation of the histidine ligand with a p$K_a$ of ~8.7. This high pH form of the protein has not been detected by our direct electrochemistry (Barker et al., 1996); instead, the currents attributed to the oxidized, protonated form decrease when the pH is increased above 8 and are undetectable at pH 9. While the midpoint potential could be slightly affected by kinetics coupled to the electrochemistry, the presence of the deprotonated, histidinate-ligated protein contributes significantly to the reduction potential measured by the equilibrium techniques employed previously (Moore et al., 1985). At pH 8.5 we obtain a potential of +165 mV, while by equilibrium measurements the value is ~+135 mV.

[2] This latter case is not perfectly rhombic since neither of the iron−sulfur bonds are normal to the heme plane; in the 2.9 Å crystal structure of bacterioferritin (Frolow et al., 1994) the sulfur atoms are both inclined toward the γ-*meso* carbon, one by 7° and the other by 11°. The heme is in fact significantly but symmetrically buckled in bacterioferritin. Care should be taken in interpreting such deviations due to the low resolution of the structure.

a variety of histidine-ligated high-spin ferric hemoproteins has allowed discrimination between true five-coordinate, histidine bound, high-spin ferric hemes and those in which the ferric ion's sixth coordination site is occupied by a water molecule. Similarly, the energy of the NIR band is also sensitive to ligand variation, but very few recorded examples are available and it is not yet clear whether or not it is as usefully diagnostic as the "630" feature. However, there is no precedent for high-spin methionine-coordinated hemes, and the MCD spectra we have obtained do not answer the question as to what occupies the sixth coordination site of the ferric ion in these high-spin forms of the variants studied here. The simple relatively intense Soret band is comparable to MCD Soret bands of the histidine/water-liganded examples studied but distinct from those of five-coordinate examples (Vickery et al., 1976; Bracete et al., 1991; Matsuoka et al., 1992; Cheesman et al., 1994). Unfortunately, histidine is the only protein residue for which the difference between 5- and 6-coordinate forms has been studied, and so it cannot be assumed that these distinctions are valid on switching to methionine ligation.

If only one methionine is ligated, which of the two possibilities is it? We have no direct evidence to answer this, but another single mutant, H102L, has a high-spin optical spectrum which is similar, but not identical, to that of H102M. This is suggestive of the possibility that Met-102 is not ligated in the H102M variant. We have not obtained EPR or MCD data for this variant. Two pieces of circumstantial evidence suggest that this Met-X ligation state is in fact not a single, stable state but may be a dynamic mixture of states. First, electrochemical analyses of the proteins which exist in the high-spin state in the oxidized form reveal that the spin-state equilibrium is coupled to the electrochemistry of the bis-methionine-coordinated species. Analysis of the electrochemical data obtained from oxidized covR98C/H102M at pH 8 in terms of the $C_rE_r$ reaction scheme (Nicholson & Shain, 1964), suggests that the kinetic parameter effecting the electrochemistry ($k_f + k_b$, Scheme 1 (Bard & Faulkner, 1980)) is about $0.1-1\ s^{-1}$ (Barker et al., 1996). At this pH, $k_b/k_f = 0.12$, and therefore the low- and high-spin states are in reasonably rapid equilibrium. Second, we have been unable to detect any hyperfine-shifted signals in the proton NMR spectra of these high-spin species (Barker & Freund, 1996). This could be explained by the interchange, on the NMR time scale, between species with vastly different spectral dispersion (i.e., different spin states).

*Reduction Potential of a Bis-Methionine-Coordinated Cytochrome.* The reversible electrochemistry of the covalent R98C/H102M protein below pH 6.5 is that of a well behaved, single electron transfer protein, and the midpoint potential of the voltammetric waves is therefore the formal reduction potential of the protein under those conditions. Under conditions in which the electrochemistry of the protein is complicated by the coupled kinetics (>pH 7), the relationship of the measured, voltammetric midpoint potential to the true reduction potential is then very dependent on the pH, the oxidation state of the protein in bulk solution, and the sweep rate (Figure 7B). Under these conditions, the measured potential only approaches the true reduction potential at high sweep rates and only with reduced protein in the bulk solution. Unfortunately, the electrochemistry of the reduced protein at high pH and high sweep rate is still not perfectly reversible (reflecting a significant rate of ligand exchange following oxidation), and hence our data only approach the true potentials. However, we believe that the midpoint potentials, measured at $0.2V\ s^{-1}$, given in Figure 7B for the reduced protein can only be at most 5−10 mV (the size of the symbol) below the true potential of the protein. We can then make the observation that the pH dependence of the midpoint potential of covR98C/H102M is similar, but not identical, to that of the wild-type protein.[1]

The potential of this bis-methionine-coordinated protein is ∼180 mV higher than that of the wild type protein at all pH values. Since the introduction of a single covalent linkage to the heme at residue 98 in the protein (R98C) with the wild type ligand arrangement slightly lowers the reduction potential (the effect varies with pH; de Oliveira, Barker, and Hill, unpublished results), the increase in reduction potential upon altering Met-His ligation to Met-Met ligation is even greater (205 mV at pH 4.8). The effect of the covalent linkage upon the reduction potential is difficult to assess at present.

This large stabilization of the reduced state of the protein by bis-methionine ligation was expected. Comparison of the redox potentials of bis-histidinyl and histidinyl-methionyl coordinated classes of cytochromes c show that potentials range from −400 to −100 mV and 0 to +400 mV, respectively (Moore & Pettigrew, 1990). Coordination of two methionyl sulfur atoms, which are good electron acceptors (Schejter et al., 1991), is expected to raise the potential even further. In the heme octapeptide model system coordination of methionine sulfur gives rise to a reduction potential more positive by 160 mV compared to that observed for imidazole coordination (Harbury & Loach, 1960; Harbury et al., 1965).

We have not been able to isolate any electrochemical response from the high-spin species with Met-X ligation. The rapid equilibration between the high- and low-spin species prior to the reduction of the low-spin species means that, at normal sweep rates, no high-spin species exists at the electrode. We would therefore only expect to observe the electrochemistry of the high-spin species at high sweep rates, but we have not found conditions under which a response is observed. If we assume that the p$K_a$ for the coordination state change in the *reduced* state of the covalent R98C/H102M is greater than 10, then we can put an upper limit of +210 mV (vs SHE at pH 7.1) on the reduction potential of the high-spin species (HS) in the thermodynamic square shown in Scheme 1 (Barker & Mauk, 1992). That is, the potential of the high-spin species is expected to be at least 180 mV lower than that of the low-spin protein. Rapid reduction of the proteins in this high-spin state could be said to be "gated", requiring ligand rearrangement prior to electron transfer. This is currently being investigated by rapid kinetic techniques.

*What are the Titratable Groups?* Interestingly, attaching the heme to the C-terminal helix at residue 98 stabilizes an oxidized species in which both methionines are ligated. However, this coordination arrangement is now dependent on the protonation state of an as yet unidentified titratable group. The measured p$K_a$ for the spectral changes must necessarily be an apparent equilibrium constant for multiple equilibria involving a protonation followed by a conformational process involving a change in coordination at the heme iron. The p$K_a$ is temperature dependent, with an enthalpyof about −12 kcal/mol (P. Barker, unpublished results), which is significantly higher than most enthalpies of ionization of

titratable groups in proteins. The only histidine in this protein, His63, has a p$K_a$ of 7.1 in both oxidation states of the wild-type protein (Moore et al., 1985). Structurally, it is difficult to see how its titration can be linked to the stability of ligation of Met102. The only possible link is that it could hydrogen bond to the Tyr101 phenolic oxygen, thereby affecting the stability of terminal helix. No such hydrogen bond is observed in the structure of the wild-type protein (Hamada et al., 1995). We have made the triple variant, R98C/Y101F/H102M, with the same covalent linkage as in covR98C/H102M, and the optical spectrum in the oxidized state is identical to that of the double variant at low and high pH values studied. It has a very similar p$K_a$ for this spectral transition. This rules out Tyr101 as the titratable residue and suggests that the involvement of His63 is unlikely. Other candidates are the many carboxylate groups in the heme binding region (especially Glu4 and Glu8 (Hamada et al., 1995)), the C-terminal carboxylate, and also the heme propionates. Identification of this titratable group through mutagenesis should allow the design of a protein with covalently-bound heme which in the oxidized state either behaves like the noncovalent, H102M protein (always high-spin) or will be low-spin at all pH values.

Since the spectroscopic properties of the reduced covR98C/H102M protein are independent of pH up to pH 9.5, the apparent p$K_a$ for a similar ligation state change in this reduced state can be assumed to be $>10$. The bulk of this change is almost certainly due to a change in the conformational equilibrium constant, and not a large change in the p$K_a$ for the actual protonation process. This system is therefore very similar to the pH-linked conformational equilibrium associated with the alkaline transition of mitochondrial cytochromes *c* (Davis et al., 1974; Barker & Mauk, 1992), in which increased stability of the fold of the ferrocytochrome drives the rearrangement of the iron ligands. It is therefore tempting to speculate that the titratable group, observed to be coupled to the spectroscopic properties of the heme in the reduced state of the single variant H102M, is the same as that observed in the oxidized state of the covalently linked, double variant.

*Comparison with Bacterioferritins.* The role of the heme group in the bacterial iron storage protein has not been established, but the 2.9 Å structure of the protein from *E. coli* has revealed that the heme pocket is formed at the interface between a pair of symmetry-related subunits which provide two equivalent methionyl residues as axial ligands. Another feature of the heme site is that it is in close proximity to a binuclear iron site. Since bacterioferritin (BFR) is the sole example of a cytochrome with bis-methionine-coordinated heme, the properties associated with the heme group in this protein may not be characteristic of this class of ligation states. Particularly, the redox potential of the heme is surprisingly low. In the absence of the iron core of the protein, the heme potential has been measured by equilibrium techniques at pH 8.0 as −225 mV (vs SHE), while in the presence of the iron phosphate core this potential drops to between −475 and −420 mV (Stiefel & Watt, 1979; Watt et al., 1986). The origin of the ∼600 mV (14 kcal/mol) difference between the reduction potential of two heme centers with the same amino acid ligands (apoBFR and covR98C/H102M) is surprising and not immediately obvious. The relatively small difference between the electronic structures of these two bis-methionine-liganded ferric hemes, as revealed by the spectroscopic parameters (*vide supra*), is unlikely to be the root of the redox differences observed. A detailed analysis of the effect of methionine ligand structure and geometry upon the redox properties of a variety of cytochromes *c* (Senn & Wüthrich, 1985) does not allow direct correlation between the two. However, the only case so far observed of a cytochrome which has different chirality at the axial methionine sulfur in each oxidation state (Senn & Wüthrich, 1983) also has a potential some 200 mV lower than the average potential for similar cytochromes *c*. Clearly, a detailed description of the ligand structure in *both* oxidation states is required to make comparisons between different systems.

Since the potential of the bis-methionine-liganded cytochrome $b_{562}$ fits into to the expected His/His < His/Met < Met/Met series, the heme redox properties in bacterioferritin must be influenced by factors other than ligand-determined electronic properties. The presence of the iron core and/or the binuclear iron site in the holoBFR protein clearly affects the potential of the heme site in this protein (Watt et al., 1986), but the influence of each of these individual iron cofactors upon the redox state of BFR has yet to be deconvoluted. Solvent exposure and Coulombic interaction with local charged residues can also dramatically affect reduction potentials, but inspection of these properties in each of the two proteins reveals nothing dramatically different between the two heme sites. If anything, the cytochrome $b_{562}$ has a more solvent exposed heme and has a higher concentration of negatively charged residues in the vicinity of the heme than does BFR. Finally, although different techniques were used to measure the potentials of these two very different protein systems, and each technique can be affected by other equilibria coupled to the redox equilibrium of interest, we do not believe this to be the reason for the large difference in measured potentials for the two heme proteins.

## CONCLUSIONS

In the following paper (Barker & Freund, 1996) it is proved that, in their reduced states, both proteins studied here have both methionines ligated to the iron. In their oxidized states, this bis-methionine ligand arrangement is destabilized relative to an alternative arrangement in which probably only one of the methionines is ligated. In this state the iron is high-spin and the structure of the ligand field may be dynamic. Both the proteins studied here have provided new, unprecedented spectroscopic information, and therefore knowledge of their structures at the heme site would enable correlation between spectroscopy and ligation state. We are currently investigating the energetics of the contribution of ligation state to the overall protein stability. The instability of the bis-methionine-coordinated ferricytochromes may be advantageous for our attempts to generate a protein which switches between two different 6-coordinate, low-spin coordination states upon oxidation state change (Barker & Mauk, 1992; Hawkins et al., 1994; Theodorakis et al., 1995; Schejter et al., 1996).

## ACKNOWLEDGMENT

We thank Drs. Ian Fearnley, MRC Laboratory of Molecular Biology, and Matthew Davison, ZENECA Pharmaceuticals, for making electrospray mass measurements.

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BI961127X