The Axial Ligands of Heme in Cytochromes: A Near-Infrared Magnetic Circular Dichroism Study of Yeast Cytochromes *c*, c_1, and *b* and Spinach Cytochrome *f*†

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ABSTRACT: Room temperature near-infrared magnetic circular dichroism and low-temperature electron paramagnetic resonance measurements have been used to characterize the ligands of the heme iron in mitochondrial cytochromes *c*, c_1, and *b* and in cytochrome *f* of the photosynthetic electron transport chain. The MCD data show that methionine is the sixth ligand of the heme of oxidized yeast cytochrome c_1; the identity of this residue is inferred to be the single conserved methionine identified from a partial alignment of the available cytochrome c_1 amino acid sequences. A different residue, which is most likely lysine, is the sixth heme ligand in oxidized spinach cytochrome *f*. The data for oxidized yeast cytochrome *b* are consistent with bis-histidine coordination of both hemes although the possibility that one of the hemes is ligated by histidine and lysine cannot be rigorously excluded. The neutral and alkaline forms of oxidized yeast cytochrome *c* have spectroscopic properties very similar to those of the horse heart proteins, and thus, by analogy, the sixth ligands are methionine and lysine, respectively.

The biological reactivity of a heme protein is controlled by a number of factors, prominent among them being the identities of the amino acid residues at the axial coordination sites of the heme iron. It thus becomes important to identify these residues. While X-ray crystallography is the method of choice for this purpose, it is usually the case that lack of satisfactory crystals precludes the use of this technique, and more generally applicable spectroscopic methods are brought into play.

In the case of low-spin ferric hemes electron paramagnetic resonance (EPR) and magnetic circular dichroism (MCD) spectroscopies have proved to be particularly useful in elucidating axial ligation. The use of EPR draws on a systematization of a large body of experimental data by Blumberg and Peisach (1972) [see review in Palmer (1982)] which provided an analysis of EPR *g* values in terms of the strength and symmetry of the ligand field that the axial ligands produce at the iron atom. This analysis led to a graphical procedure by which the chemical nature of these axial ligands could be deduced [see, for example, Kulmacz et al. (1987)].

MCD spectra can be obtained over a wide range of wavelengths. However, it has been shown by Stephens and coworkers (Cheng et al., 1973; Stephens et al., 1976; Rawlings et al., 1977) that the near-infrared (IR) region (800–2000 nm) is of particular value in the study of axial ligation. Low-spin ferric hemes generally exhibit electronic transitions in this range that can be assigned to porphyrin (π) to metal (3d) charge-transfer excitations. Since the energies of the metal d orbitals are a function of the axial ligands of the Fe atom, the energies of the near-IR transitions are a function of the axial ligation. Axial ligation, when unknown, can thus be inferred by a comparison of near-IR transition energies to those of hemes of known coordination. The near-IR region is superior to the visible and ultraviolet for this purpose since the latter are dominated by porphyrin π–π^* transitions whose energies depend only weakly, and in a complex manner, on the axial ligands. MCD is to be preferred to conventional absorption spectroscopy because the latter is made difficult by solvent and protein vibrational absorption in this spectral region. Furthermore, in those cases where both low- and high-spin heme states are present, MCD enables the nature of the ground state associated with a particular electronic transition to be identified.

The earliest application of near-IR MCD to the study of axial ligation in low-spin ferric hemes involved the high-pH forms of cytochrome *c'* (Rawlings et al., 1977) and made use of near-IR MCD spectra of a variety of forms of myoglobin, hemoglobin, and cytochrome *c*. This study both illustrated the potential of the technique in this area and made evident the need for reference spectra of a much wider range of heme ligation types. Since that time, near-IR MCD spectra of a number of heme proteins and model compounds have been reported by Thomson and co-workers (Gadsby et al., 1987, and references cited therein).

Most low-spin ferric heme proteins exhibit EPR g_z values less than 3.1 (Palmer, 1982), and the above methods have been most commonly applied to such proteins. However, there exists an important group of such heme proteins with values for g_z which are significantly larger than 3.1 and which can approach the theoretical limit of 4.0 (Palmer, 1985). Proteins in this family are often called "highly anisotropic low-spin" or HALS heme proteins, though an important source of this large anisotropy is likely to be a relatively high symmetry of the ligand field (Palmer, 1985). Important examples of HALS proteins are to be found among cytochromes that are integrally associated with membranes. Prominent among these are the cytochromes *b* and c_1 from complex III (bc_1) of the mitochondrial electron-transfer chain (Orme-Johnson et al., 1974; Siedow et al., 1978) and cytochrome *f* from the analogous complex of the photosynthetic electron-transfer chain (Siedow et al., 1980). While cytochromes c_1 and *f* are usually compared to the structurally well-characterized cytochrome *c* (Takano et al., 1972) and hence assumed to have histidine–methionine

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coordination, in neither case in the 695-nm absorbance signature of the Fe–methionine bond readily apparent (Siedow et al., 1980; J. S. Siedow and G. Palmer, unpublished observations), and the EPR g_z values of cytochromes *f* and c_1 are about 3.5 (Siedow et al., 1978, 1980), significantly larger than the value of 3.06–3.07 found with beef and horse heart cytochromes *c* (Salmeen & Palmer, 1966; Lambeth et al., 1973). Furthermore, in their comparisons of cytochromes c_1 from *Paracoccus denitrificans*, *Rhodobacter capsulatus*, and yeast and bovine heart mitochondria, Kurowski and Ludwig (1987) aligned the four protein sequences for maximum sequence homology. By so doing the classical -C-X-X-C-H- sequence of residues diagnostic of the *c*-type heme binding site was consistently identified. However, in this same sequence comparison no conserved methionine can be found, implying that this amino acid is not involved in heme ligation. The cytochrome *c* heme binding site is also found in the amino acid sequences of a variety of cytochromes *f* (Alt & Herrmann, 1984); however, in apparent contrast to cytochrome c_1, the cytochromes *f* also exhibit a conserved methionine close to the carboxyl terminus. The bis-heme mitochondrial cytochromes *b* are usually compared with well-characterized cytochromes *b* [e.g., b_5 (Mathews et al., 1972)] and assumed to have bis-histidine coordination. This is supported by model building based on sequence analysis (Widger et al., 1984). From studies of model compounds (Carter et al., 1981) the unusual low-field *g* values, approaching 3.8, that are found with the mitochondrial cytochromes *b* have been rationalized in terms of a "strained" conformation (Carter et al., 1981; Palmer, 1985).

In view of the extreme importance of these proteins in cellular function and the uncertainties that remain with respect to their axial ligation, we have studied the near-IR MCD of yeast cytochromes c_1 and *b* and spinach cytochrome *f*. In addition, we have also examined the spectral properties of yeast cytochrome *c* at both neutral and alkaline pH. The data lead to the unexpected conclusion that cytochrome c_1 indeed has histidine–methionine coordination, while cytochrome *f* is almost certainly a histidine–lysine derivative. Our data also support the conclusion that both hemes of yeast cytochrome *b* possess histidine–histidine coordination though histidine–lysine coordination at one heme center cannot be totally excluded. Finally, yeast cytochrome *c* is shown to possess axial coordination identical with that of the horse heart cytochrome *c* at both neutral and alkaline pH.

After submission of this paper a study of the charlock, rape, and woad cytochromes *f* appeared, using NMR, EPR, and MCD (Rigby et al., 1988). This independent, parallel study arrives at the conclusion that these cytochromes *f* possess histidine–lysine coordination, in accord with our conclusion for the spinach protein.

Materials and Methods

Yeast mitochondrial cytochromes *b* and c_1 were obtained by using a newly developed procedure (D. Simpkin and G. Palmer, unpublished results) in which complex III prepared by the method of Siedow et al. (1978) was delipidated by the procedure of Salerno et al. (1986). The delipidated material was then chromatographed on octyl-Sepharose, which binds cytochrome *b* more strongly than cytochrome c_1, and the two proteins were easily separated by varying the ionic strength. Cytochromes *b* and c_1 were then further purified by ammonium sulfate fractionation and chromatography on (diethylaminoethyl)cellulose; the final products contained 29.7 nmol of heme B/mg of protein and 32.3 nmol of heme C/mg of protein, respectively. Yeast cytochrome *c* (type VIIIB) and spinach cytochrome *f* were purchased from Sigma. The high-pH form of cytochrome *c* was prepared by raising the pH of a sample of protein to 11.9. Each sample was exchanged into D_2O (Sigma) by several cycles of dilution in the respective buffer (prepared in D_2O) followed by concentration using a Centricon microconcentrator (Amicon). The concentrations of the samples ranged from 0.5 (cytochrome *b*) to 2.5 mM (cytochrome *c*). All samples except the high-pH cytochrome *c* were in 50 mM potassium phosphate and 1 mM EDTA, pD 7.4, with 0.5% dodecyl maltoside. The high-pH sample was in 0.1 M potassium triphosphate and 1 mM EDTA, pD 12.3. The samples were shipped to Los Angeles for MCD measurements in a shipping container that had been prechilled in liquid nitrogen. Following MCD spectroscopy the samples were returned to Houston and portions which had been used for the MCD measurements together with unused portions were examined by EPR. There were no observable differences between the used and unused materials.

EPR spectra were recorded at 12 K with a Varian E-6 X-band spectrometer; experimental conditions are shown in the legend to Figure 1. In some samples multiple low-spin EPR signals were observed, although one signal was predominant in each case. The relative amounts of the low-spin species present in some samples were established by recording the low-field region of the spectrum at 100 μW, resolving the individual absorption features manually, and determining the area of each component by "cutting and weighing". These integrals were then corrected for the transition probability of each species by multiplying by *W*, which was calculated from the formula

$$W = 105.6 + 50g_z + 5.9g_z^2$$

obtained by fitting the tabulated data of De Vries and Albracht (1979).

MCD spectra were measured with an infared circular dichroism spectrometer described elsewhere (Osborne et al., 1973; Nafie et al., 1976; Stephens & Clark, 1979; Devlin & Stephens, 1987). For these studies light was provided by a 2-kW carbon rod source, a $CaCO_3$ Glan polarizer (Karl Lambrecht Corp.) and a 52-kHz octagonal CaF_2 photoelastic modulator (Hinds Intl.) were used, and the light was focused by a *f*1.7 CaF_2 lens (Janose Technology) onto a 1 mm diameter InSb detector (Santa Barbara Research Co.) mounted in a closed cycle refrigerator (CTI) and cooled to ≈30 K. The magnetic field was obtained by using an Oxford Instruments SM5 Spectromag split-coil superconducting magnet and was operated at ±3 T. Samples were placed in cells of ≈1-mm path length, maintained at 18 °C. MCD spectra were obtained at both +3 and −3 T unless the signal-to-noise ratio was high, when only +3-T data were recorded. Prior to the MCD measurement the protein samples were examined by visible absorption spectroscopy. To samples that were not fully oxidized was added excess ammonium persulfate (Tanaka et al., 1978); this oxidant was used because it and its products give no MCD in the near-IR. Because MCD spectra were recorded at room temperature, any artifacts (Gadsby et al., 1987) arising from the use of glassing agents are avoided.

Results

The 12 K EPR spectra in the heme low-spin region of the several proteins studied are shown in Figure 1 at both 20-mW and 100-μW incident microwave power. In the cases of cytochromes c_1, *f*, and *b* multiple low-spin resonances are observed with the HALS species dominating the experimental spectra at both microwave powers. At 100 μW no low-spin species is saturated while at 20 mW the HALS species remain

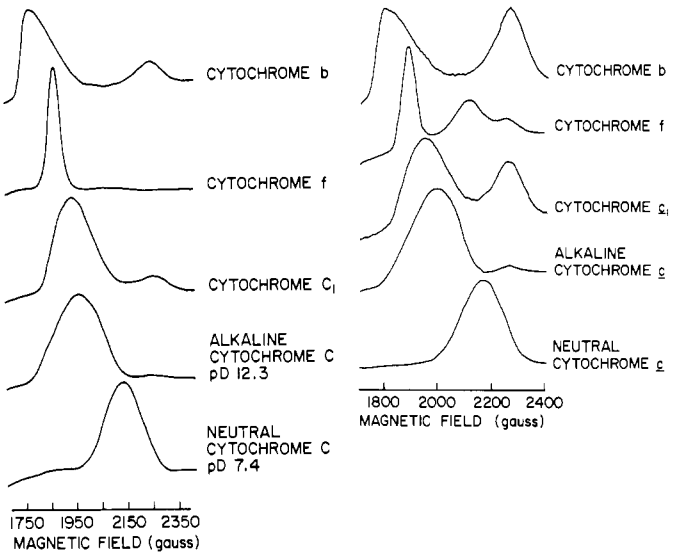

FIGURE 1: EPR spectra of ferricytochromes as indicated. Left: Data recorded at 20 mW. Right: Data recorded at 100 μW. Other instrumental conditions: temperature, 12 K; modulation amplitude, 10 G; frequency, 9.234 GHz.

Table I: Proportions of the Low-Spin Species in the Cytochromes Studied

protein	low-field g value (g_z)	% abundance[a]
yeast cytochrome c, pD 7.4	3.06	100
yeast cytochrome c, pD 12.3	3.32	100
yeast cytochrome c_1, pD 7.4	3.45	84
	2.95	16
spinach cytochrome f, pD 7.4	3.53	77
	3.06	19
	2.95	4
yeast cytochrome b, pD 7.4	3.69	89
	2.95	11

[a] Expressed as the proportion of the total low-spin area, the individual peaks being weighted by their transition probability (see Materials and Methods).

unsaturated, but the minority species, which possess smaller values for g_z, are significantly saturated. The relative populations of the various low-spin species that were present were quantified by integration of the g_z absorption features recorded at 100 μW followed by a correction for differences in transition probability as described by De Vries and Albracht (1979); the results are given in Table I. The g_z values obtained for cytochrome b (3.69), cytochrome f (3.53), cytochrome c_1 (3.45), and neutral cytochrome c (3.06) are similar to those reported previously for these or related proteins (Tsai & Palmer, 1982; Siedow et al., 1978, 1980; Salmeen & Palmer, 1966; Lambeth et al., 1972); the value of g_z for alkaline yeast cytochrome c (3.32) has not been reported previously. It should be noted that these absorption features are sufficiently broad that precise location of the EPR maxima is difficult. It should also be noted that each of these samples showed some $g = 6$ high-spin heme signals together with some $g = 4.3$ signals. These EPR species are not normally seen in these materials, and we attribute their presence to the deuteration procedure.

The MCD spectra of the several proteins are shown in Figure 2. In all cases the spectra are typical of low-spin hemes. Two principal features of the same sign are observed, the lower energy feature exhibiting the stronger MCD. In no case is any feature attributable to a high-spin heme detectable. The peak wavelengths, λ_1 and λ_2, of the two main MCD features are given in Table II.

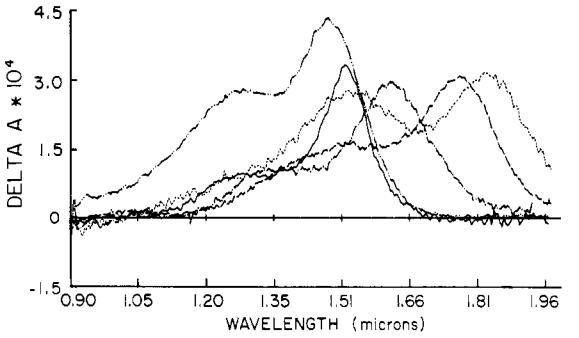

FIGURE 2: Near-infrared MCD spectra of ferricytochromes as indicated. The spectra for cytochromes b and c_1 are shown three times the actual size. Temperature, 18 °C. (—) Cytochrome f (pD 7.4); (--) cytochrome c (pD 7.4); (-·-) cytochrome b (pD 7.4); (-··-) cytochrome c (pD 12.3); (---) cytochrome c_1 (pD 7.4).

Table II: Near-IR MCD Maxima of the Cytochromes Studied

protein	λ_2[a]	λ_1[a]
yeast cytochrome c, pD 7.4	1521 (6576)	1756 (5694)
yeast cytochrome c, pD 12.3	1286 (7774)	1464 (6829)
yeast cytochrome c_1, pD 7.4	1528 (6545)	1838 (5441)
spinach cytochrome f, pD 7.4	1256 (7964)	1506 (6640)
yeast cytochrome b, pD 7.4	1376 (7270)	1605 (6230)

[a] Units are nanometers; values in parentheses are in units of cm^{-1}.

Discussion

Near-IR MCD has been measured for a range of low-spin ferric heme proteins and iron–porphyrin derivatives. In general, a monosignate MCD spectrum with the maxima is observed, with the maximum at low energy invariably being the more intense. The MCD is sometimes sharper at low temperatures and in a few cases exhibits additional structure. For a selection of cases where the axial ligation is adequately characterized, the wavelength of the principal MCD maximum, λ_1, is given in Table III. In some cases the MCD was recorded at room temperature; in other cases it was measured near 4.2 K. Where measurements are available at both temperatures, peak wavelengths differ slightly, as expected, due to changes in the vibronic envelope with temperature. From theory (Cheng et al., 1973; Stephens et al., 1976), two low-energy electronic transitions are expected, arising from excitations from a_{2u} and a_{1u} porphyrin π molecular orbitals (D_{4h} symmetry designation) into the hole in the t_{2g} metal 3d orbitals. Both transitions are predicted to have the same anisotropy ratio ($\Delta\epsilon/\epsilon$). The principal near-IR MCD features can therefore be assigned either to the 0–0 bands of two separate electronic transitions or to the 0–0 and 0–1 bands of a single electronic transition. In the following discussion, the principal focus will be on the energy of the lowest transition, and the uncertainty in the precise spectroscopic assignment will not be of importance.

In the studies reported here we have measured the near-IR MCD of oxidized yeast cytochrome c at neutral and alkaline pH values, together with oxidized yeast cytochromes c_1 and b and oxidized spinach cytochrome f; all these measurements were made at room temperature and had the objective of identifying the intrinsic axial ligands in these proteins. The MCD was measured at room temperature rather than at low temperatures for several reasons. First, it is easier. Second, the physiological temperature is more closely approximated. Third, low-temperature measurements require the addition of high concentrations of glassing agents such as glycerol or ethylene glycol; there are clear examples where such reagents substantially modify the geometric and electronic structure of the active site. For example, in a recent study ethylene

Table III: EPR g_z Values and Near-IR MCD Maxima (λ_1) for Selected Axial Coordination Types

coordination	g_z	λ_1[a]	example[b]	ref
Met–His	3.05	1750/1725	horse heart cyt *c*	Rawlings et al., 1977; Gadsby, et al., 1987
	3.2	1800/1690	*P. aeruginosa* cyt c_{551}	Foote et al., 1984; Gadsby et al., 1987
	3.3 (or 3.0)	1870/1740	*P. aeruginosa* cyt c_{551} peroxidase	Foote et al., 1984
	3.04	1860	*E. coli* cyt b_{562}	Moore et al., 1985
	3.01	1750	*P. aeruginosa* nitrite reductase	Sutherland et al., 1986
Met–His$^-$	2.79	1550	*E. coli* cyt b_{562} (high pH)	Moore et al., 1985
His–His	2.95	1600	methemoglobin + Imid	Rawlings et al., 1977; Sievers et al., 1983a
	2.93	/1600	metmyoglobin + Imid	Gadsby & Thomson, 1982
	2.96	1510	horse heart cyt *c* + Imid	Rawlings et al., 1977; Sievers et al., 1983a
	3.2–2.7 (several)	1505/1500	*D. vulgaris* cyt c_3	Foote et al., 1984; Dervartanian & LeGall, 1978
	3.0 (or 3.3)	1500/1480	*P. aeruginosa* cyt c_{551} peroxidase	Foote et al., 1984
	3.03	/1610	leghemoglobin + Imid	Sievers et al., 1983a
	2.98	/1620	leghemoglobin + 1-MeImid	Sievers et al., 1983a
	2.76	1600	leghemoglobin	Sievers et al., 1983a
	2.96	1510	*W. succinogenes* nitrite reductase	Blackmore et al., 1987
	3.77	1545	*P. aeruginosa* formate dehydrogenase	Godfrey et al., 1987
	3.52	1480	bis(1,2-diMeImid)–OEP	Gadsby & Thomson, 1986
His–His$^-$	2.80	/1350	metmyoglobin + Imid (high pH)	Gadsby & Thomson, 1982
	2.82	1350	leghemoglobin + Imid (high pH)	Sievers et al., 1983a
	3.17, 2.94	1365	lactoperoxidase	Sievers et al., 1983b
His–Lys	3.4	1590	hemoglobin + lysine	Rawlings et al., 1977
	3.50	1480/1465	horse heart cyt *c* (high pH)	Gadsby, et al., 1987
	3.38	1550	leghemoglobin + butylamine	Gadsby et al., 1987
Lys–Lys	3.69	1320	bis(butylamine)–OEP	Gadsby & Thomson, 1986
His–Tyr$^-$	2.65	1160	leghemoglobin + phenol	Sievers et al., 1983a
His–OH$^-$	2.59	1050	metmyoglobin (high pH)	Eglington et al., 1983

[a] In nanometers. Values before and after the slash are for ≈4 K and room temperature, respectively. [b] All data are at neutral pH unless otherwise specified. Abbreviations: OEP, ferric octaethylporphyrin; Imid, imidazole; 1-MeImid, 1-methylimidazole; 1,2-diMeImid, 1,2-dimethylimidazole.

glycol was found to significantly alter and complicate the EPR spectrum of horse heart cytochrome *c* (Gadsby et al., 1987). In our experiments we have observed the monosignate MCD spectra typical of low-spin ferric hemes in all cases; we did not detect any of the bisignate MCD typical of high-spin hemes. Our spectra thus support the conclusion that all of the cytochromes studied exist predominantly in low-spin states. We also recorded the EPR spectrum of each sample that was examined in the MCD spectrometer. Since the EPR is not detectable at room temperature, these spectra were necessarily recorded at low temperature. In those cases where a single low-spin EPR spectrum was observed (cytochrome *c* at neutral and alkaline pH) we assign this spectrum to the species responsible for the room temperature MCD. The remaining proteins exhibited small amounts of minority species. Although these minority species are very conspicuous in the low-power EPR spectra (Figure 1), this is a consequence of the differences in transition probability which lead to an increased amplitude for species that have a smaller *g* anisotropy. Because the HALS species appear to be a consequence of a "strained" heme coordination (Carter et al., 1982), minor perturbations are expected to relax the structural constraints responsible for the unusual heme environment, thus facilitating conversion to non-HALS species and a corresponding decrease in g_z; this process can occur without any change in the identity of the axial ligands. Whether the perturbation responsible for these effects arises from the freezing process or from some chemical treatment remains to be established.

The EPR and MCD spectra of yeast cytochrome *c* are very similar to those reported for the corresponding protein from horse heart at both pH values studied (Lambeth et al., 1973; Gadsby et al., 1987). At pH values close to 7 the low-field *g* value for both proteins is 3.05–3.06. The lowest energy MCD maximum (λ_1) is at 1725 nm in the horse heart protein while in the yeast protein it is slightly red-shifted, to 1756 nm; the higher energy maximum (λ_2) is at 1550 and 1521 nm, respectively. These λ_1 values are diagnostic of histidine–methionine coordination of the heme iron (see Table III). Thus, as expected, the ligand field at the heme iron must be very similar in the two proteins. In view of their very similar primary sequence (Margoliash et al., 1961; Narita et al., 1963), and hence tertiary structure, this is hardly surprising.

The alkaline form of horse heart cytochrome *c* has long been suspected to be a form of the protein in which the methionine attached to the heme iron has been replaced by lysine (Lambeth et al., 1973). The recent near-IR MCD study of Thomson and co-workers further substantiates this conclusion (Gadsby et al., 1987). Although lysine-79 was originally suggested to be the relevant residue, a body of chemical data (Bosshard, 1981) has thrown doubt on that suggestion, and the specific lysine residue remains to be identified. The EPR g_z values are 3.32 and 3.50, λ_2 = 1286 and 1280 nm and λ_1 = 1464 and 1465 nm, for the yeast and horse heart proteins, respectively. These similarities in the EPR and MCD spectra of the alkaline forms of yeast and horse heart cytochromes *c* make it clear that the change in ligation which occurs when horse heart cytochrome *c* is made alkaline also occurs with the yeast protein. Yeast *iso*-1 cytochrome *c* conserves 11 of the 19 lysine residues present in the horse heart protein (Margoliash et al., 1961; Narita et al., 1963); thus the location of the ligating lysine residue is not substantially constrained by a comparison of amino acid sequences. Unfortunately, data on the existence of the alkaline transition are lacking for the vast majority of other proteins from this family.

Yeast cytochrome c_1 exhibits values of 1528 and 1838 nm for λ_2 and λ_1, respectively (Figure 2, Table II). The value of λ_1 immediately demonstrates the presence of histidine–methionine axial ligation. Although λ_1 is somewhat greater than that found with the mitochondrial cytochromes *c*, it is important to appreciate that a range of λ_1 values are to be associated with any given coordination type; in the case of histidine–methionine coordination the available values range from 1690 to 1870 nm (Table III). The value of λ_1 found with cytochrome c_1 is within this range and closest to the value of 1860 nm found for the cytochrome *b*-562 from *Escherichia coli*. Thus despite its unusually large value for g_z and the difficulty in resolving the 695-nm absorption, the very low energy of the lower energy porphyrin-to-metal charge-transfer

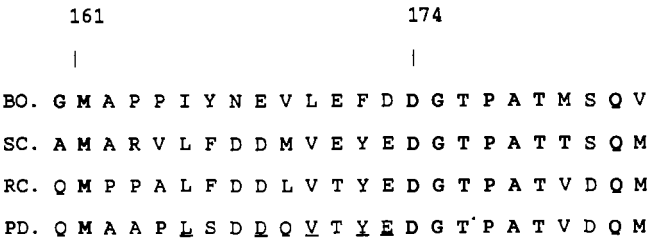

FIGURE 3: Alignment of partial amino acid sequences of the polypeptides for cytochrome c_1 from cow (BO) (Wakabayashi et al., 1980), yeast (SC) (Sadler et al., 1984), *P. denitrificans* (PD) (Kurowski & Ludwig, 1987), and *R. capsulatus* (RC) (Gabellini & Sebald, 1986). Totally conserved residues are shown in bold type. Semiconserved residues are underlined.

band makes it clear that methionine is the sixth ligand of the heme iron in yeast mitochondrial cytochrome c_1.

This conclusion is at variance with the sequence alignment of Kurowski and Ludwig (1987), who compared the sequences of cytochromes c_1 isolated from *P. denitrificans*, *R. capsulatus*, yeast, and cows; their comparisons did not reveal any conserved methionine. However, on further examination of these sequences we have found that all four proteins contain the strictly conserved sequence DGTPAT (which begins at residue 174 in the yeast sequence, toward the carboxyl terminus) and that upon realigning these polypeptide sequences using this segment a conserved methionine is found at residue 161 (Figure 3). We propose that it is this methionine that is the sixth ligand to the heme iron. We also infer that the proximal histidine is in the neutral form; were the histidine present as imidazolate, the EPR g_z value would probably be much smaller, about 2.8, and λ_1 would be found near 1550 nm (Table III).

By contrast, although spinach cytochrome *f* exhibits a value for g_z which is similar to that of cytochrome c_1, its near-IR MCD is shifted to substantially higher energy (Figure 2, Table II). Indeed both g_z and the wavelengths of λ_1 and λ_2 of cytochrome *f* are much closer to those found with the alkaline form of cytochrome *c* than to the spectral parameters observed with neutral cytochrome *c*. This similarity supports the conclusion that cytochrome *f*, like alkaline cytochrome *c* (Gadsby et al., 1987; Lambeth et al., 1973), contains histidine and lysine as the ligands of the heme iron. It must be recognized, however, that very similar values for g_z and λ_1 are found with the model compound, bis(1,2-dimethylimidazole)–Fe(III)–octaethylporphyrin (Carter et al., 1981; Gadsby & Thomson, 1986). By virtue of the steric hindrance imposed by the 2-methyl group this compound is recognized as having a strained coordination at the heme, and it is this strain that is responsible for the unusually large value for g_z. Thus on the basis of these spectral data alone it is not possible to resolve the alternative of strained histidine–histidine coordination from histidine–lysine coordination. Fortunately, from the six available sequences of cytochrome *f* (Hauska et al., 1988) it is readily established that there are several completely conserved lysines but only one conserved histidine. This histidine is associated with the *c*-type cytochrome binding site and must thus be the proximal ligand. We conclude that the second axial ligand must be lysine. Interestingly, the completely conserved lysine-145 aligns closely with the position of the conserved methionine of cytochrome c_1 identified above, and it is most plausible that this lysine is the heme ligand. An important caveat, however, is that the lack of a second fully conserved histidine rests on the substitution found in the pea cytochrome *f* (His → Tyr), and it cannot be completely excluded that this is a (single base) sequencing error. Consequently our conclusion should be viewed as provisional. The available sequences for cytochrome *f* (Hauska et al., 1988) also reveal a conserved methionine that is located two residues from the carboxyl terminus. Were the methionine a ligand, the near-IR MCD data would require that the coordination be methione–histidinate (Table III); this combination is excluded by the large value for g_z observed with this protein. Furthermore, according to hydropathy analyses for this protein (Cramer et al., 1987), the heme binding site and carboxyl terminus are located on different sides of the membrane, and it thus seems most unlikely that this methionine residue could be involved in heme ligation.

Histidine–lysine coordination in cytochrome *f* was proposed earlier by Siedow et al. (1978) on the basis of visible–UV, MCD, and EPR measurements. Further support for this assignment has very recently been presented by Rigby et al. (1988), who used NMR, MCD, and EPR to characterize cytochrome F from charlock, rape, and woad. The NMR spectra were found to lack the classical resonances attributable to an axial methionine. In the reduced form, two upfield-shifted single-proton resonances were observed; these were assigned to the β-CH_2 of lysine though, in principle, any methylene group adjacent to a ligating atom would provide the NMR result. MCD was reported at 4.2 K but not room temperature and for the visible in addition to the near-IR. The near-IR maximum was reorted to be at 1520 nm, close to the value that we have obtained using spinach cytochrome *f*; from these data Rigby et al. (1988) drew the same conclusion regarding the axial ligands in this protein. The EPR spectra were also similar to those reported here and previously (Siedow et al., 1978). It should be noted that the EPR spectra reported by Rigby et al. were obtained at high microwave power (20 mW), thus suppressing the contribution of any saturating non-HALS components. Rigby et al. also propose that the original interpretation of the EPR spectrum of cytochrome *f* (Siedow et al., 1978) should be revised, with g_y being approximately 1.7 and not 2.1 as suggested originally. This conclusion is based on an, as yet, unpublished theory of near-IR MCD intensities and remains to be firmly substantiated. It should also be noted that Davis et al. (1988) found that the resonance Raman spectrum of turnip cytochrome *f* exhibits a porphyrin mode at 1533 cm^{-1}, similar to that found in alkaline cytochrome *c* but 10 cm^{-1} to lower energy than the comparable mode in both neutral cytochromes *c* and c_1, and concluded that lysine must be the sixth heme ligand in this protein. At this time, however, this Raman criterion is not based upon a sufficient range of reference compounds to allow a reliable assessment of its validity.

As with cytochrome *f* the values of g_z, λ_1, and λ_2 for yeast cytochrome *b* can be modeled equally well by both histidine–histidine and histidine–lysine reference compounds, and again we cannot resolve the alternative coordinations using our spectroscopic data alone. However, more than 20 amino acid sequences are available for the polypeptide of cytochrome *b*, and alignment of these sequences (Hauska et al., 1988) reveals that only one lysine, residue 288 (yeast numbering), is completely conserved among all 20 species (lysine-238 is replaced by arginine in two species). Thus we can conclude that, at most, only one of the two protoheme centers in cytochrome *b* can have histidine–lyine coordination and that the other center is undoubtedly histidine–histidine. Lysine-336 is located at the amino terminus of helix VII of Crofts' model (Crofts et al., 1988). As spinach cytochrome b_6 is too short to contain this helix, it is probable that lysine-288 should also be considered to be not fully conserved, though in view of the structural differences between mitochondrial cytochrome c_1

and plant cytochrome *f* reported above, a comparison of the photosynthetic and mitochondrial cytochromes *b* may be misleading. The same logic can be applied to the single conserved methionine (residue 157) present in mitochondrial cytochrome *b* but which is not conserved in b_6, particularly as the plant protein exhibits EPR spectra similar to those of the mitochondrial cytochrome (Salerno et al., 1983). If the sequence of cytochrome b_6 is indeed included in the comparison, one is forced to conclude that bis-histidine coordination is present at both heme centers, as originally proposed by Widger et al. (1984).

CONCLUSIONS

Although EPR has enjoyed considerable use in the past 15 years as a tool to diagnose heme coordination types, it has become apparent that the magnitude and anisotropy of the *g* tensor, which is manipulated in the Blumberg–Peisach analysis, become an unreliable indicator of coordination type as the ligand field symmetry at the heme approaches axiality ($V \ll \Delta$); under these conditions the magnitude of g_z increases upon decreasing Δ or upon decreasing V. Decreases in Δ arise through changes in the ligand field strength of the coordinating atom, while decreases in V can arise either because of special geometric relationships between heme ligands, for example, a pair of histidine residues which are oriented so that they are mutually perpendicular, or when the distal ligand is constrained with a noncoordinating lone pair parallel to the plane of the proximal histidine. Both conditions can lead to values for g_z that are much larger than 3.06 (see Table III). This sensitivity to two factors complicates the reliability of EPR in making assignments of heme ligands. By contrast, the energies of the near-IR ligand-to-metal charge-transfer excitations are much less sensitive to ligand orientation, and as a consequence, the energies of the near-IR MCD bands are a more accurate indicator of ligand type than are the EPR *g* values. Even so, the available data make it clear that neither method, taken either singly or together, is definitive.

Both the EPR and MCD techniques rely upon comparisons with reference low-spin systems of known coordination. Unfortunately, this reference set is limited in scope, and there are no unambiguously defined structural types other than histidine–histidine and histidine–methionine, though models derived from the addition of exogenous ligands to heme proteins are likely to be of the coordination anticipated. Thus, in part, a cyclic situation has arisen in which inconclusively characterized systems are subsequently adopted as defined standards. This difficulty becomes particularly serious when one is faced with the task of identifying an atypically coordinated heme within the constraints of a small set of prototypes.

As it stands, it is clear that EPR is unable to reliably identify ligand types when g_z is greater than ca. 3.1. In these cases the MCD technique is able to establish methionine–(neutral) histidine coordination, but other possibilities for ligation remain ambiguous. The use of amino acid sequence data to identify completely conserved potential ligands asists in the interpretation of the spectra but can usually be criticized on the grounds that the relevant spectral data are lacking for those protein species that are being compared. It is thus clear that there is a need for both additional spectroscopic probes able to define ligand types and additional low-spin heme proteins with well-defined coordination; providing these resources is an important immediate challenge.

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Plastocyanin Cytochrome *f* Interaction†

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ABSTRACT: Spinach plastocyanin and turnip cytochrome *f* have been covalently linked by using a water-soluble carbodiimide to yield an adduct of the two proteins. The redox potential of cytochrome *f* in the adduct was shifted by −20 mV relative to that of free cytochrome *f*, while the redox potential of plastocyanin in the adduct was the same as that of free plastocyanin. Solvent perturbation studies showed the degree of heme exposure in the adduct to be less than in free cytochrome *f*, indicating that plastocyanin was linked in such a way as to bury the exposed heme edge. Small changes were also observed when the resonance Raman spectrum of the adduct was compared to that of free cytochrome *f*. The adduct was incapable of interacting with or donating electrons to photosystem I. Peptide mapping and sequencing studies revealed two sites of linkage between the two proteins. In one site of linkage, Asp-44 of plastocyanin is covalently linked to Lys-187 of cytochrome *f*. This represents the first identification of a group on cytochrome *f* that is involved in the interaction with plastocyanin. The other site of linkage involves Glu-59 and/or Glu-60 of plastocyanin to as yet unidentified amino groups on cytochrome *f*. *Euglena* cytochrome *c*-552 could also be covalently linked to turnip cytochrome *f*, although with a lower efficiency than spinach plastocyanin. In contrast, a variety of cyanobacterial cytochrome *c*-553's and a cyanobacterial plastocyanin could not be covalently linked to turnip cytochrome *f*.

Cytochrome *f* (cyt *f*)[1] of the photosynthetic electron-transport chain functions between the two photosystems to reduce plastocyanin (PC) using electrons from the Rieske iron–sulfur protein of the cytochrome *b6/f* complex as a reductant (Bendall, 1982; Hauska et al., 1983). Cyt *f* and PC are functional analogues of cytochrome c_1 and cytochrome *c*, which function in the mitochondrial electron-transport chain. In some algae and cyanobacteria, a small *c*-type cytochrome, cyt *c*-553, replaces PC under certain growth conditions (Wood,

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[1] Abbreviations: Chl, chlorophyll; cyt *f*, cytochrome *f*; PC, plastocyanin; PC–cyt *f*, covalently linked adduct between plastocyanin and cytochrome *f*; cyt *c*-553, cytochrome *c*-553; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; SDS, sodium dodecyl sulfate; PS I, photosystem I; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; MV, methylviologen; CNBr, cyanogen bromide; TFA, trifluoroacetic acid.

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