Magnetic Circular Dichroism Studies of Low-Spin Cytochromes. Temperature Dependence and Effects of Axial Coordination on the Spectra of Cytochrome *c* and Cytochrome b_5

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Abstract: Magnetic circular dichroism (MCD) spectra are reported for oxidized and reduced cytochromes *c* and b_5 in the near-uv and visible spectral regions. At room temperature the oxidized cytochromes exhibit Soret MCD spectra characteristic of hemichromes, as observed with low-spin complexes of ferrimyoglobin (preceding paper in this issue). This suggests that the spin state of the iron is more important in determining the MCD in this region and for this redox state than are differences in the protein crevice. Extremes of pH caused changes in the MCD spectra which were consistent with previously proposed spin-state changes. Visible MCD spectra of hemichrome derivatives differing in axial coordination (native, alkaline, and complexes with azide, cyanide, and imidazole) are distinctive, while derivatives with similar iron ligands (cytochrome *c*-imidazole, myoglobin-imidazole, and cytochrome b_5) show visible MCD spectra closely resembling one another. This suggests the utility of an empirical examination of the MCD in the visible region of oxidized hemoproteins for identifying iron ligands. The reduced cytochromes exhibit visible MCD spectra very similar in shape to one another and typical of other hemochromes. Thus the visible region can serve as a marker for the presence of reduced, low-spin heme; this effect is illustrated by a comparison of the difference MCD spectrum of reduced vs. oxidized chloroplast cytochromes *f*, b_{559}, and b_{563} with a computer-synthesized curve based on reduced cytochrome *c*. The Soret MCD spectra, however, are very different for reduced cytochromes *c* and b_5 and do not resemble other low-spin ferrohemoproteins; this spectral region therefore may be sensitive to the mechanism of heme binding by the polypeptide. Low-temperature MCD spectra of the oxidized cytochromes show the presence of Faraday *C* terms, indicative of paramagnetic iron-porphyrin interaction, associated with the porphyrin π-π^* transitions and charge-transfer transitions. Reduced cytochrome *c* exhibits no temperature effects in the Soret region, but sharpening of the visible bands gives rise to more intensive *A* terms in that region.

In the preceding paper the magnetic circular dichroism (MCD) of a series of myoglobin derivatives was reported.[1] The MCD spectra were found to be sensitive to the electronic state of the heme, and the shape and intensity of the spectra could be correlated with the redox state, the spin state, and the axial ligation of the iron. Here we report investigation of these effects in cytochromes *c* and b_5. The heme moiety of each of these proteins is low spin in both the oxidized and reduced states, but changes in spin state and axial coordination of the iron can be effected by extremes of pH and the presence of additional iron-binding ligands. Thus the results obtained with the oxygen storage and transport protein, myoglobin, can be extended to hemoproteins involved in electron transport systems, and the generality of the conclusions reached with the former hemoprotein can be assessed. In addition, since the heme environment or crevice is different for the two cytochromes, the effects of the protein structure on the MCD of two functionally related hemoproteins can be studied.

Several reports on the magneto-optical activity of native cytochrome *c*[2-10] and of native cytochrome b_5[10-12] have appeared, but the effects of alteration of the protein conformation on the MCD spectra have not been explored in any detail. X-Ray crystal structures of cytochromes *c*[13] and b_5[14] are known, however, and numerous other techniques have been used to characterize conformational changes that can be effected in solution. Thus changes in the MCD spectra should be interpretable in terms of an altered heme-protein interaction. A better understanding of the effects of the protein structure on the MCD spectra is necessary if the results of MCD studies of cytochromes in situ[11,12,15,16] are to be interpreted.

Materials and Methods

Crystalline horse heart cytochrome *c* (Type VI) was obtained from Sigma. Purified cytochrome b_5 obtained by tryptic treatment of calf liver was the generous gift of Dr. P. Strittmatter. The cytochromes were oxidized with potassium ferricyanide and reduced anaerobically with sodium dithionite. Concentrations of the native proteins were determined according to published extinction coefficients.[17,18]

The effects of pH on the absorption spectra of ferricytochrome *c*[19] and ferricytochrome b_5[20-22] were similar to those reported previously. The binding of azide,[23,24] cyanide,[23,25] and imidazole[24,26] to cytochrome *c* was monitored spectrophotometrically.

The methods of recording absorption and MCD spectra and the low-temperature techniques are described in the preceding paper.[1] Spectra reported at low temperatures are not corrected for volume changes in the glass-forming solvents due to difficulties in monitoring the volume in the small spectrophotometric cell used. In a modified cell we observed a linear decrease in volume of 7 to 8% on lowering the temperature of a mixture of glycerol and 0.5 *M* potassium chloride (6:4 v/v) from 22 to −130°; for a mixture of equal volumes of potassium glycerophosphate, glycerol, and water a decrease of approximately 5% was found for the same range. A Corning Model 110 pH meter was used for pH measurements.

Results

Ferricytochromes. **Soret Region.** MCD spectra of the Soret band of cytochrome *c* obtained at 24 and −75° are shown in Figure 1. The room-temperature curve is similar in shape and intensity to that reported by previous workers.[4,5,8,10] On lowering the temperature by 99° no significant changes in shape occurred, but the intensity increased approximately 60% at the 413-nm trough and 40% at the 401-nm peak. Since a 50% increase is expected on the basis of a Boltzmann distribution for this temperature change, we conclude that both spectra are composed almost exclusively of Faraday *C*-type terms. The inset to Figure 1 shows that

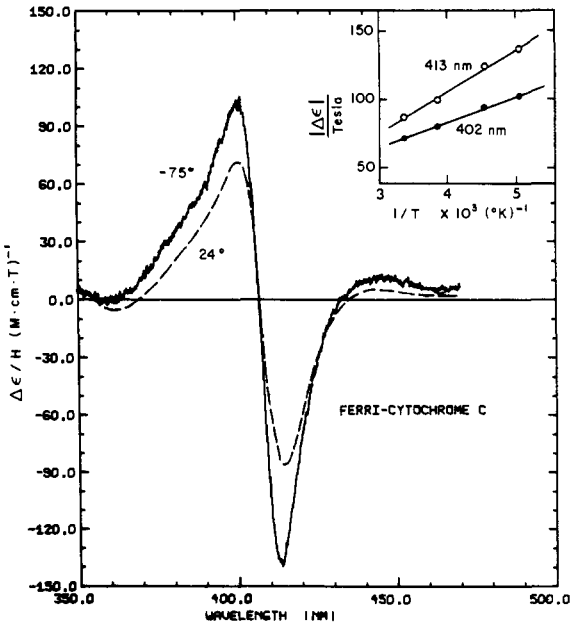

Figure 1. The effect of temperature on the Soret MCD of ferricytochrome c. The sample was 3.3×10^{-5} M in a solvent of glycerol and 0.5 M KCl (6:4 v/v) at pH 7.3; pathlength = 0.23 cm, field = 0.9 T; 2 passes, one for each field direction, were averaged. The inset shows the temperature dependence of the trough and peak extrema MCD intensity.

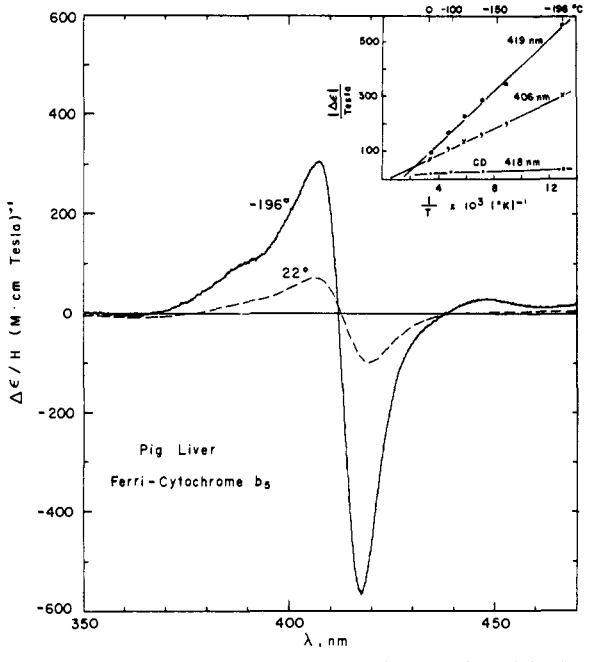

Figure 2. The effect of temperature on the Soret MCD of ferricytochrome b_5. The sample was 3.1×10^{-5} M in a solvent of potassium glycerophosphate, glycerol, water (1:1:1 v/v/v), pH 7.2, path = 0.23 cm, field = 0.9 T, 4 passes averaged. The inset shows the temperature dependence of the negative CD intensity (vertical scale $\Delta\epsilon$) of the 418-nm trough as well as that of the MCD extrema.

the increase is linear with the reciprocal of the absolute temperature. The effects on the MCD of changes in temperature as small as 10° are readily measured.

The results of the effects of a wider range of temperature on the Soret band MCD of cytochrome b_5 are shown in Figure 2. The curve obtained at room temperature resembles that observed by others.[10-12] As with cytochrome c, the shape of the MCD was not altered upon lowering the temperature, but the intensity increased linearly with the reciprocal of the absolute temperature. Evidence against large band narrowing or significant solvent contraction contributions to the intensity increase was obtained by the measurement of the natural circular dichroism (CD) simultaneous with the MCD measurements.[1,27] The inset of Figure 2 shows that the intensity of the Soret CD peak at 418 nm exhibits very little temperature dependence. Thus the Soret MCD spectrum of cytochrome b_5 consists predominantly of C terms. However, the fact that extrapolation of the temperature dependence of the MCD intensity of the trough near 419 nm does not approach zero at infinite temperature (Figure 2, inset) indicates the presence of other, albeit weaker, Faraday effects in this region.

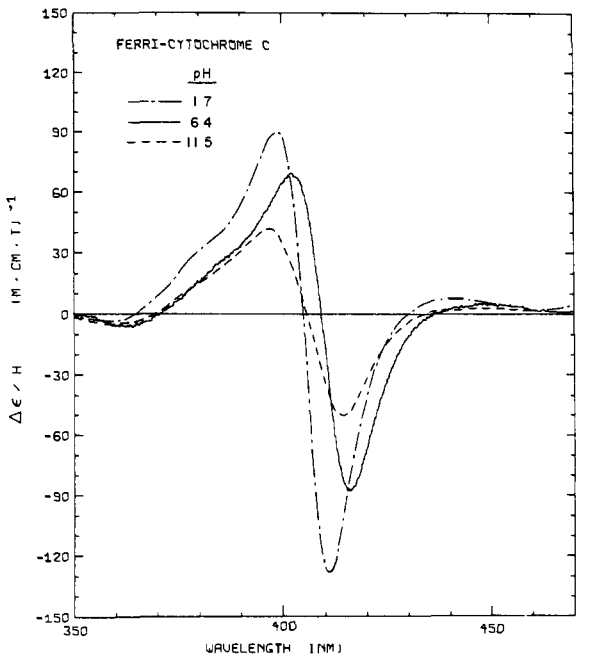

Figure 3. The effect of pH on the MCD of ferricytochrome c. The sample was 10^{-5} M in 0.6 M KCl, path = 1.0 cm; field = 1.4 T, 6 passes were averaged for each spectrum, temperature = ambient (near 22°).

The effects of different pH on the Soret region MCD of cytochrome c are given in Figure 3. At alkaline pH changes in axial ligation of the heme are proposed to occur, but the iron remains in the low-spin state.[19,28-34] The Soret absorption peak shifts from 409 to 406 nm and increases in intensity by about 13%. This change is paralleled by a shift in the zerocrossing of the Soret MCD from 409 to 405 nm and an increase in intensity by approximately 37%. The shape of the MCD spectrum is unchanged and is typical of a low-spin, ferric heme complex. The increase in the MCD intensity may reflect a combination of the increase in extinction at alkaline pH and the slight narrowing of the band as evidenced by the decrease in the splitting of the MCD extrema (peak and trough positions) from 13.5 to 12.0 nm. The MCD intensity is expected to increase linearly with the absorption and as the inverse square with the band width.

Under acidic conditions cytochrome c is extensively denatured, and changes in both axial ligation and spin state of the iron occur.[19,28,29,35,36] The protein-iron bonds are broken, but the heme moiety remains covalently attached to the protein via thioether linkages condensed from vinyl side chains of the porphyrin and cysteine residues of the protein. The iron is converted from a low-spin ($S = \frac{1}{2}$) to a high-spin ($S = \frac{5}{2}$) form. In the presence of excess chloride, which binds to the iron, a mixed-spin equilibrium complex is formed,[28,37] and the Soret MCD intensity is expected to decrease. This is what is observed (Figure 3). If we assume that the low-spin form present has an absorption spectrum

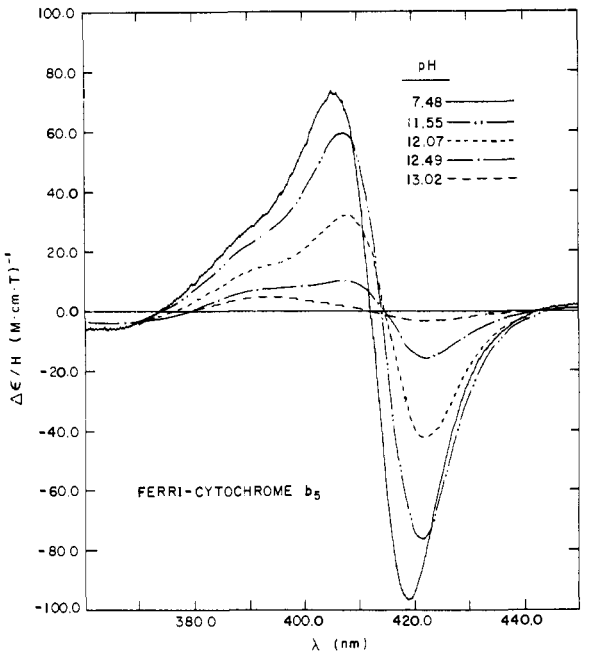

Figure 4. The effect of alkaline pH on the MCD of ferricytochrome b_5. The sample was 1.1×10^{-5} M in 0.1 M KCl, path = 1.0 cm, field = 1.4 T, 4 passes were averaged at each pH value, temperature = ambient.

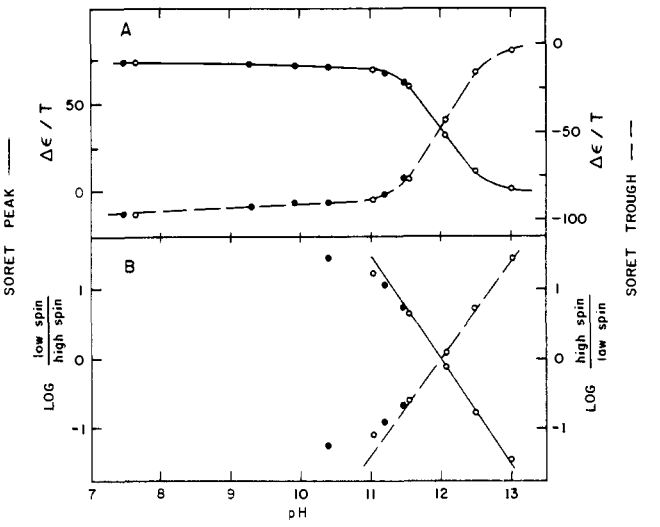

Figure 5. MCD spectrophotometric titration of ferricytochrome b_5. The data are taken from Figure 4.

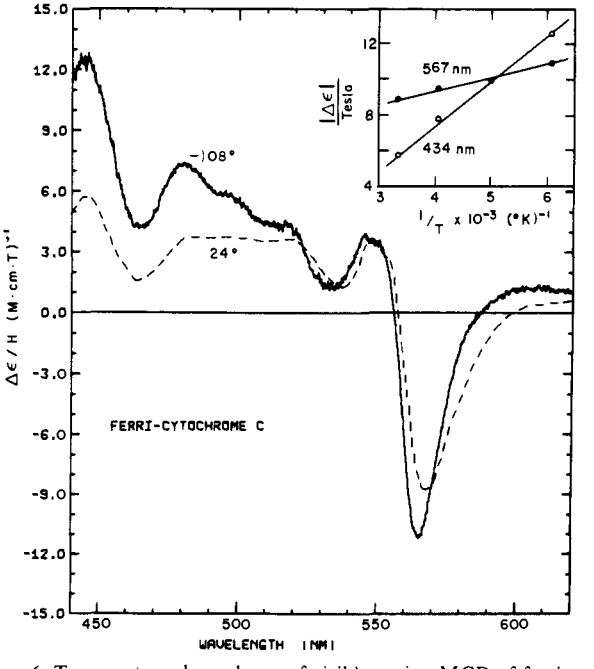

Figure 6. Temperature dependence of visible region MCD of ferricytochrome c. The sample concentration was 4×10^{-4} M, other conditions were as in Figure 1 except that 4 passes were averaged.

similar to that of native and alkaline cytochrome c, then the intensity of the MCD at pH 1.7 suggests that about 59% of the low pH complex is low spin. This can be compared with the intermediate spin value ($S = 3/2$) determined by Boeri et al.[37] from magnetic susceptibility measurements.

The effects of alkaline conditions on the MCD of ferricytochrome b_5 were also investigated. MCD spectra at various pH values are shown in Figure 4. The intensities of the MCD at the positive and negative extrema are plotted as a function of pH in Figure 5A. The peak near 407 nm and the trough near 420 nm both decrease throughout the range measured, indicating a conversion from low to high spin.[1] Evidence for the presence of high-spin derivative formation has also been obtained from EPR[20,22] and absorption[20-22] measurements. The midpoint for the transition observed in the MCD occurs at pH 12.0 (Figure 5A). Assuming that the MCD recorded at pH 7 corresponds to 100% low spin and that the completely high-spin derivative formed at pH greater than 13 will make essentially no contribution to the spectra in this region, we have plotted the ratio of the high- to low-spin forms as a function of pH in Figure 5B. The pK_a is 12.0 calculated from either extremum for the spin-state transition. The slope of the curve obtained from the data for the peak is 1.4 and that from the trough is 1.5, indicating that more than a single proton is involved in the conformational change.

Deviations in the linearity of the log ratio plots (Figure 5B) occur when less than 10% of the high-spin form is expected to exist. This behavior is also apparent in the gradual slope of the data from pH 7 to 10 in Figure 5A. Such deviations might reflect the intermediate formation of a different low-spin form. Evidence for the presence of such an intermediate can also be inferred from the lack of an isoelliptic point in the MCD spectra (Figure 4). Instead, the zero-crossing shifts from 412.5 to 415 nm as the pH is raised. This red shift is more than one-half completed by pH 11.55, indicating a pK_a for the transition lower than this value. Multiple low-spin forms are also apparent in the low-temperature EPR spectra of alkaline solutions of cytochrome b_5.[20,22]

Visible Region. The temperature dependence of the MCD of the visible absorption bands of cytochrome c was also investigated. Figure 6 shows the MCD spectra obtained at 24 and −108°. Complex changes were observed on the long-wavelength side of and near the Q_0 band. The MCD near 590 nm becomes positive while the trough near 567 nm becomes more negative as the temperature is lowered. This sign pattern is reversed from that observed in the Soret region of ferricytochromes c and b_5 (Figures 1 and 2), but it resembles the temperature effects found for the visible bands of ferrimyoglobin cyanide.[1]

There is little structure in the MCD associated with the Q_v bands (530 nm), but several extrema exhibiting strong temperature dependencies are observed at shorter wavelength. The peak at 480 nm increases 90% and the peak at 444 nm increases 120% over the range studied. A Boltzmann factor of 1.8 is expected for these temperatures and hence these MCD features are predominantly C terms. We have previously attributed bands in this region of heme

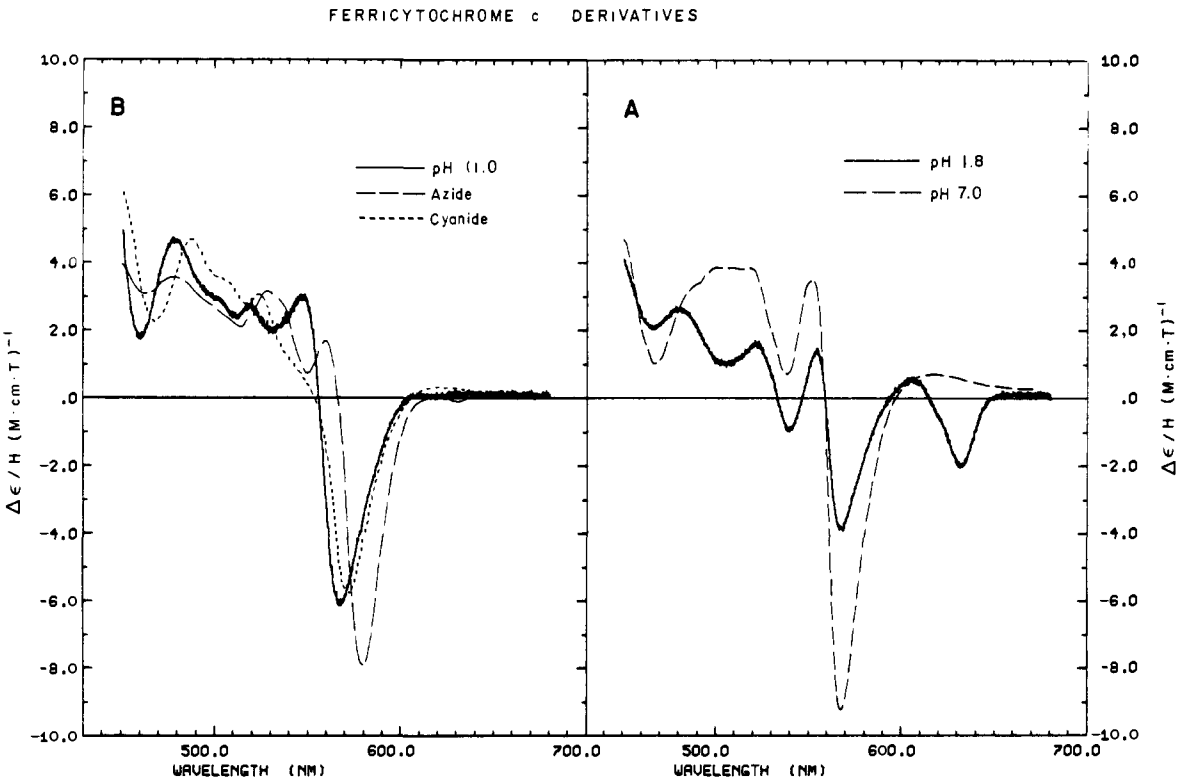

Figure 7. MCD spectra of ferricytochrome *c* derivatives. The sample conditions were as described in Figure 3 except that the protein concentrations used were ca. 5 to 9 × 10^{-5} *M*.

complexes, between the major visible (Q) and near-uv (Soret) bands, to charge-transfer transitions involving the porphyrin and the iron.[1] The sensitivity of these bands to the nature of the axial ligands will be reported below.

The iron of native cytochrome *c* is coordinated to the protein via the thioether sulfur of a methionine side chain and the imidazole nitrogen of a histidine residue.[13] This form of the reduced cytochrome is stable over a wide pH range, but the oxidized cytochrome undergoes reversible changes at pH values less than 4 or greater than 7. The effect of acid pH and chloride anion on the visible MCD of ferricytochrome *c* is given in Figure 7A. The neutral pH curve is similar to that reported by Sutherland and Klein[8] but differs from that reported by Risler and Groudinsky.[10] On lowering the pH from 6.8 to 1.8 a trough appears at 632 nm with a zerocrossing at 615 nm, and the *A* term with a zerocrossing at 557 nm is considerably weakened. These changes correspond to the appearance of a charge-transfer band at 620 nm and the decrease of the Q_0 band intensity in the absorption spectra, respectively. They reflect the shift from a purely low-spin complex to a low spin-high spin equilibrium which occurs upon replacement of the relatively strong field sulfur and nitrogen ligands by chloride. The MCD band shape at wavelengths less than 538 nm, or to the blue of the Q_v band, is also altered. These differences in the MCD could reflect changes in the intensities of absorption bands in this region arising from spin-state changes, or they could reflect shifts in band positions of charge-transfer transitions arising from the replacement of the amino acid ligands by chloride. A combination of these effects is also possible and complicates the interpretation.

Alteration of the axial coordination of ferricytochrome *c* without changing the spin state of the iron can, however, be achieved by several methods. Under alkaline conditions the methionyl sulfur is believed to be replaced by a nearby lysyl amino group[29-34] forming a new type of low-spin complex. The MCD spectrum of the derivative formed at pH 11.5 is

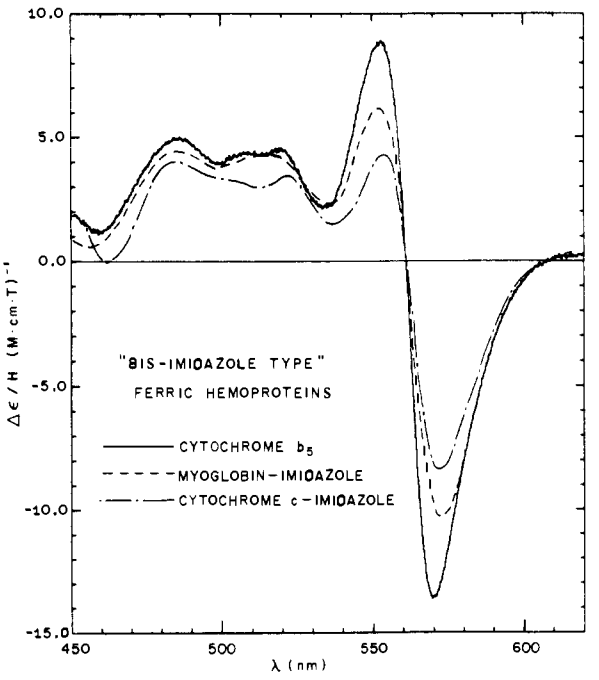

Figure 8. Comparison of the visible MCD spectra of bis(imidazole) coordinated ferrihemoproteins. The cytochrome *c* and myoglobin spectra have been red and blue shifted, respectively; see text. All spectra were recorded at ambient temperatures.

shown in Figure 7B. At wavelengths greater than 520 nm, or in the region of the Q_0 and Q_v bands, the MCD of the alkaline derivative resembles that of the native form seen at neutral pH (Figure 7A). The main feature is a Faraday *A* term corresponding to the Q_0 transition. At shorter wavelengths, however, considerable differences in the shape of the MCD spectra are evident. If the MCD in this region arises from charge-transfer transitions involving the por-

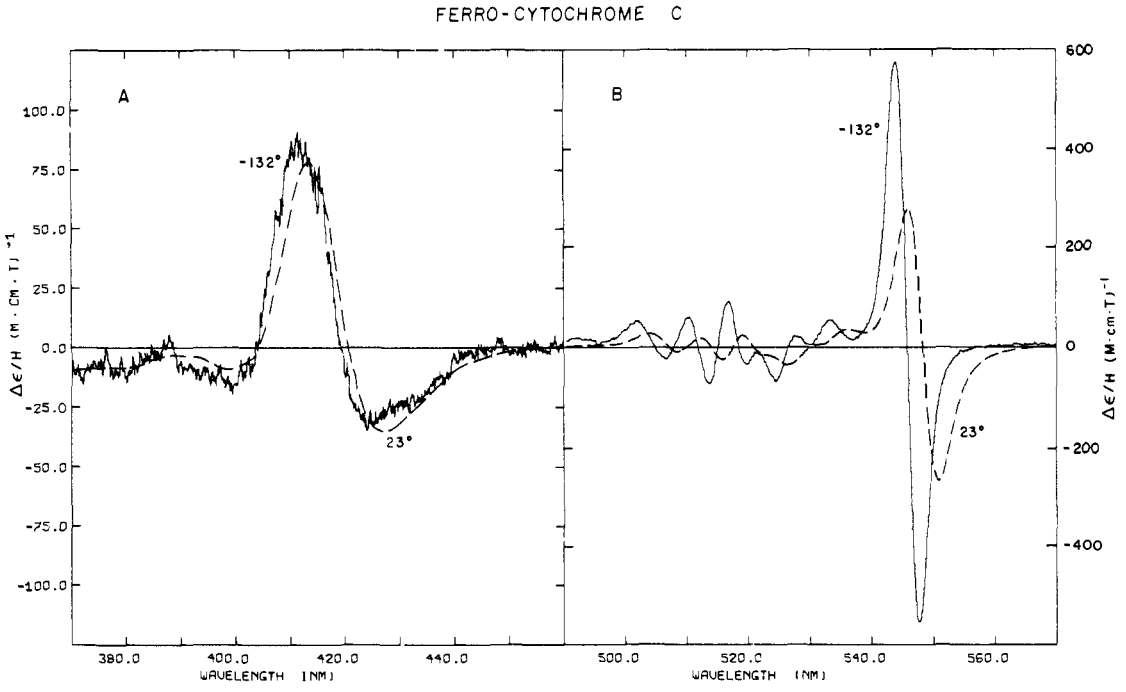

Figure 9. Temperature dependence of the MCD spectrum of ferrocytochrome *c*. The sample was 3.2×10^{-5} *M* in a solvent of potassium glycerophosphate, glycerol, 0.1 *M* sodium phosphate (1:2:1 v/v/v) at pH 6.8, path = 0.23 cm, field = 0.9 T, 2 passes were averaged.

phyrin and the metal,[1] changes in the nature of the axial ligands would be expected to alter the shape and/or position of these bands.

We have tested this proposal further by the addition of exogenous ligands which can bind to the iron of ferricytochrome *c*. High concentrations of azide,[23,24] cyanide,[23,25] and imidazole[24,26] will displace methionine as the heme's sixth ligand and form new low-spin complexes. The visible MCD spectra of the former derivatives are also given in Figure 7B. The azide complex exhibits an *A* term at 566 nm associated with the Q_0 band and a weak trough at 630 nm associated with a weak charge-transfer transition in this region. In the 440 to 530-nm region the shape of the MCD is quite different from that of native ferricytochrome *c*, but resembles that of alkaline cytochrome *c* in which a nitrogen has also replaced the sulfur of methionine in bonding to the iron. The visible MCD spectrum of ferrimyoglobin azide[1] also shows similar features. The cyanide complex does not show a well-resolved *A* term for the Q_0 transition, but this is probably due to the broadness of this band, as described for ferrimyoglobin cyanide.[1] Below 530 nm the cyanide complex differs from the native protein but shows some similarities to the alkaline and azide forms.

The MCD spectrum of the imidazole complex of ferricytochrome *c* is given in Figure 8 along with the spectra of two other hemoproteins in which the iron is coordinated to two imidazole nitrogens. The cytochrome *c* imidazole curve has been red shifted by 3 nm and the myoglobin imidazole curve blue shifted by 2 nm to make the zerocrossings of the Q_0 transition *A* terms coincide with that of cytochrome b_5 and facilitate comparison of the shape of the MCD. Unlike the derivatives which differ in axial coordination, these spectra show striking similarities in the 450 to 530-nm region.

Ferrocytochromes. Reduced cytochromes *c* and b_5 are both low-spin ($S = 0$) hemoproteins and hence the effects found with paramagnetic derivatives are not expected. Indeed, the room-temperature MCD spectra in the visible region of ferrocytochrome *c*[5,8] and ferrocytochrome b_5[11,12] are similar and exhibit an intense *A* term associated with the Q_0 transition, which is typical of other low-spin ferrohemoproteins and other metalloporphyrins (see ref 1). The Soret region spectrum of reduced cytochrome *c*[5,8] does not resemble that of cytochrome b_5,[11,12] however, nor are the Soret MCD spectra of these cytochromes similar to those of any other ferrohemoproteins.[1] We have therefore measured the temperature dependence of the MCD of ferrocytochrome *c* in order to determine whether evidence for additional transitions, such as observed with carbonmonoxymyoglobin,[1] can be found. Earlier absorption measurements of cytochromes suggested that band narrowing can lead to intensity changes in the visible region, but that band narrowing and intensification are not significant in the Soret region.[38]

Table I. Magnetic Circular Dichroism of Reduced Cytochrome *c* and Cytochrome b_5

Cytochrome *c*		Cytochrome b_5[a]	
	$\Delta\epsilon/H$, $(M\ \text{cm T})^{-1}$		$\Delta\epsilon/H$, $(M\ \text{cm T})^{-1}$
400	(−7)	415	(−9)
404	(0)	420	(0)
413	(+78)	426	(+20)
420	(0)	432	(0)
427	(−33)	438	(−6)

[a] Reference 12.

Soret Region. MCD spectra in the near-uv region of reduced cytochrome *c* at 23 and −132° are shown in Figure 9A, and the room-temperature results are summarized in Table I. No significant effects of lowering the temperature on the intensity or the shape of the MCD were observed. At both temperatures, the MCD resembles a somewhat asymmetric *A* term with a zerocrossing red shifted by about 4 nm from the Soret absorption maximum. The presence of Faraday *B* terms in addition to the expected *A* term could account for deviations from the normal absorption-derivative MCD shape.

MCD results for the Soret region of reduced cytochrome b_5 are also given in Table I. The shape of the MCD is similar to that of cytochrome *c*, but the intensity is much weaker, even though the absorption coefficient for cytochrome b_5 (ϵ_{423} 171 000) is greater than that for the cytochrome *c*

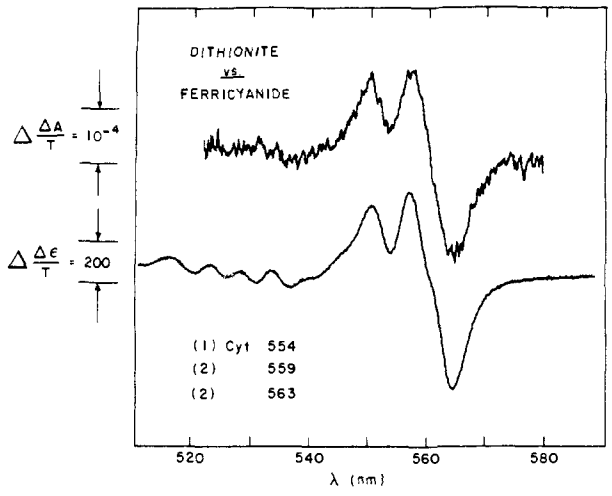

Figure 10. Difference MCD spectra of reduced vs. oxidized chloroplasts (upper curve) and "cytochromes c" (lower curve) at ambient temperature. See text for explanation.

(ϵ_{416} 129 000). In addition, the entire curve is more red shifted relative to the absorption maximum than is observed for cytochrome c and bears little resemblance to the derivative of the Soret absorption band.

Visible Region. Figure 9B shows the visible region MCD spectra of the same sample of ferrocytochrome c. Unlike for the Soret band, lowering the temperature produces large changes in the MCD of the Q bands. The zerocrossing of the Q_0 band shifts from 548.2 nm at 23° to 545.7 nm at −132°, and the peak-to-trough splitting decreases from 4.8 to 3.6 nm. The ratio of the reciprocal of squares of these bandwidths should give rise to an increased intensity of the A term by 80% due to narrowing alone. The actual ratio found for the two temperatures is close to this predicted value, i.e., a 100% increase. A Boltzmann redistribution upon lowering the temperature 155° would be expected to increase the MCD by an *additional* factor of 1.1; hence we conclude that C terms are not important here. Sharpening and blue shifts in the MCD of the Q vibrational bands are also apparent in Figure 9B.

Cytochrome Mixtures. The MCD associated with the Q_0 band of low-spin, reduced cytochromes appears to be due solely to a Faraday A type term, since the excited state for this transition is approximately X-Y degenerate. Therefore, it is expected that all cytochromes will exhibit similarly shaped MCD curves in this region, except for small shifts in the zerocrossing associated with changes in the position of the Q_0 band and perhaps MCD intensity differences reflecting differences in either the bandwidth of the Q_0 transition or the orbital angular momentum of the excited state. For low-spin, reduced heme complexes the Q_0 transition is usually quite narrow and the porphyrin π electrons can be considered "nearly free",[5,8] so that intense A terms are found.

For these reasons sharp S-shaped MCD bands in the 550 to 565-nm region observed in crude, heterogeneous preparations of chloroplasts,[15] microsomes,[11,12,16] and mitochondria[39] have been attributed to reduced cytochromes. The MCD spectra of chloroplast lamellae are dominated by the MCD of chlorophylls, but Breton and Hilaire were able to observe a small A term near 555 nm upon the addition of sodium dithionite.[15] The exact shape and position of the A term was difficult to assess, however, because the signal was superimposed upon the intense, negative B term arising from chlorophyll A in this region.

We have overcome this difficulty by plotting MCD difference spectra of data which have been digitally stored in a computer.[37] Figure 10 shows the MCD difference spectrum of a chloroplast preparation which has been treated with sodium dithionite to reduce cytochromes or with potassium ferricyanide to oxidize cytochromes. The shape of the MCD difference curve does not resemble a single A term but could arise from the overlap of several A terms displaced in wavelength. A similarly shaped MCD curve could be synthesized by shifting the cytochrome c curve shown in Figure 9B by 4, 9, and 13 nm to the red and then summing the resulting spectra. The lower curve shown in Figure 10 was obobtained when such cytochrome c-like MCD curves with Q_0 band absorption maxima at 554, 559, and 563 nm were summed in a ratio of 1:2:2. The curves produced by adding other ratios or curves shifted to different degrees yielded less satisfactory fits to the experimental results. Other methods of analysis for chloroplast cytochromes have shown the presence of one c-type cytochrome with a Q_0 band at 554 nm, and two b-type cytochromes with Q_0 band maxima at 559 and 563 nm in a ratio of 1:2:2.[40]

From the intensity of the difference MCD spectrum we can calculate the cytochrome concentrations if it is assumed that the chloroplast cytochromes have an MCD intensity similar to that of cytochrome c. On this basis it is estimated that cytochrome 554 is approximately 0.3 μM and cytochromes 559 and 563 are 0.6 μM; since the chlorophyll concentration in these particles is $\approx$120 μM, the ratio of these cytochromes to chlorophyll is about 1:2:2:400. This is similar to the ratio proposed for the photosynthetic unit.[40]

Since the computer-synthesized MCD curve may not represent a unique fit to the experimental curve, MCD spectra of the individual cytochrome components were isolated by poising the oxidation-reduction potential of the system to sequentially reduce the cytochromes. MCD difference spectra were then generated for different redox potential ranges. The results of such a MCD redox titration are in agreement with the previously proposed cytochrome composition of chloroplast lamellae[40] and support the comparison of interpretation of the experimental curve in terms of the synthetic curve shown in Figure 10.

Discussion

Previous studies have established the sensitivity of the magneto-optical activity of cytochromes c and b_5 to the oxidation-reduction state of the heme.[2-12] The changes seen in the MCD during redox changes are thought to reflect primarily changes in the bandwidth, extinction, and position of the absorption bands[8] as no alterations in axial ligation or conversion to a high-spin form occur in the native proteins. In the experiments here we have investigated the effects of spin state and axial coordination, within a given redox state, for these cytochromes. The effect of temperature on the MCD was also studied as a prerequisite to assigning Faraday C terms in the spectra.

In the oxidized state both cytochromes exhibited similar S-shaped MCD spectra in the Soret region. The temperature dependence of the MCD intensity, but not the shape, establishes the origin as C terms. These must arise from paramagnetic effects due to the iron and hence should be sensitive to spin state rather than axial coordination.[1] This appears to be the case since the Soret MCD of all ferrihemeproteins investigated possessing an appreciable amount of low-spin heme show similarly shaped curves regardless of the identity of the axial ligands. This is observed for imino-thioether (cytochrome c), bis-imino (cytochrome b_5 and ferrimyoglobin imidazole), imino-amino (alkaline cytochrome c), imino-carbon (myoglobin cyanide), and imino-oxygen (ferrimyoglobin aquo and hydroxide) coordination of the iron. Differences in axial coordination as well as heme environment can alter the exact position and the bandwidth of the spectrum. The major effect observed in

this region, however, is the change in intensity with changes in spin state. The intense S-shaped MCD can thus serve as a marker for *hemichromes* and can be utilized to determine the amount of low-spin component(s) present. In this report cytochromes *c* and b_5 were shown to lose their hemichrome character at extreme pH values. This MCD signal has also been used to determine that microsomal membrane bound cytochrome b_5 also exists essentially in the completely low-spin form at ambient temperatures[12] and to confirm earlier reports that cytochrome P-450$_{cam}$ is converted from low to high spin upon substrate binding.[11,12] The Soret MCD should also be useful for monitoring spin-state changes in other oxidized hemeproteins caused by effector molecules.

The visible region MCD of oxidized cytochrome *c* also shows temperature-dependent bands indicative of the presence of *C* terms. In addition new MCD bands, not resolved in the absorption spectra, are observed for both cytochromes. As was found for ferrimyoglobin,[1] these bands are sensitive to both the spin state of the heme and the nature of the axial ligands to the iron. The MCD spectra obtained with the low-spin derivatives suggest that an analysis of the visible region may be used to identify and possibly monitor ligand changes in ferrihemeproteins. It is noteworthy that no visible MCD spectra of the low-spin heme complexes studied here or in the preceding paper (ref 1), which are restricted to nitrogen, thioether, and carbon ligands, resemble the spectrum of ferricytochrome P-450$_{cam}$, in which mercaptide coordination has been proposed.[11,12]

In the reduced state Faraday *C* term effects, which are sensitive to the electronic structure of the heme iron, were not expected since both cytochromes *c* and b_5 normally exist in the low-spin, diamagnetic state. In the case of the Q_0 or α band this appears to be true, since cytochromes *c* and b_5 exhibit sharp *A* terms similar to those observed in other ferrocytochromes and low-spin reduced hemoproteins. High-spin reduced hemoproteins such as deoxymyoglobin,[1] deoxyhemoglobin,[41] and cytochrome P-450$_{cam}$[12] do not exhibit a similar band. Thus this band appears to be diagnostic for the presence of *hemochromes*. Because of the intensity of the Q_0 MCD *A* term, concentrations of hemochrome as low as 10^{-7} *M* can be measured; because the band is sharp and distinctive in shape, small amounts of hemochrome can be detected in the presence of a large excess of other magneto-optically active species. Thus, this band is useful for identifying dilute cytochromes in situ[11,12,15,16,39] and for detecting small percentages of reduced heme in an oxidized sample.[42] The *A* term associated with the hemochrome Q_0 band may also be useful for monitoring spin-state equilibria in ferrous heme complexes. Attempts to study temperature effects in such systems, however, will be complicated by both band sharpening effects in low-spin derivatives (shown here for ferrocytochrome *c*) and *C* terms which may be present in the paramagnetic high-spin forms. This problem was encountered in the interpretation of the visible region MCD of deoxymyoglobin.[1]

The Soret region MCD spectra of reduced cytochromes *c* and b_5, on the other hand, are quite different from one another. Neither cytochrome exhibits the simple *A* term seen in oxy- and carbonmonoxymyoglobin[1] and hemoglobin[41] or carbonmonoxycytochrome P-450.[11,12] Because the low-temperature MCD spectrum of ferrocytochrome *c* reported here provides no evidence for the presence of *C* terms, Faraday *B* terms may be uniquely important in this spectral region. Since *B* terms arise from magnetic field induced mixing of ground and excited states, the differences in the MCD spectra of cytochromes *c* and b_5 could reflect the differences in the energy separation and polarization of the electronic transitions for the two types of cytochromes in this region. It will be of interest to compare the MCD spectra of other cytochromes and hemoproteins in the Soret region.

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