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Near-Infrared Magnetic Circular Dichroism of Cytochrome *c'* †

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ABSTRACT: The near-infrared magnetic circular dichroism (MCD) of *Rhodospirillum rubrum, Chromatium vinosum,* and *Rhodopseudomonas palustris* cytochromes *c'* are reported. The spectra of the reduced protein are very similar to those of deoxymyoglobin. The spectra of the oxidized proteins in the pD range 1–13 can be analyzed on the basis of four species A, B, C, and D. The existence of nine species, reported in a recent electron paramagnetic resonance study, is not substantiated. The MCD spectra support the assignment of B as high spin and C and D as low spin. The MCD of species A is close to that of high-spin proteins and does not support the recently proposed assignment of a mixed high- and intermediate-spin ground state for this species. The energies of the near-IR electronic transitions of all four oxidized species point to axial ligation via oxygen, assuming histidine to be the opposite axial ligand. Unfortunately, insufficient model compounds with ligation by carboxyl or hydroxyl moieties exist to enable more precise assignments.

The cytochromes *c'* are atypical heme proteins (for recent reviews, see Kamen and Horio, 1970; Kamen et al., 1972). In the reduced state, their optical spectra and magnetic susceptibility are myoglobin-like, but the ligand binding characteristics are substantially different (Taniguchi and Kamen, 1963). In the oxidized state at neutral pH, their optical spectra, magnetic susceptibility, and EPR[1] most resemble high-spin ($S = 5/2$) acid metmyoglobin. However, the room-temperature magnetic moment is below the usual high-spin range and the low-temperature EPR differs from that expected (Ehrenberg and Kamen, 1965). The resonance Raman (Strekas and Spiro, 1974) and Mössbauer (Moss et al., 1968) spectra are also unusual. In addition, the anion binding behavior is unlike that of acid metmyoglobin. The magnetic properties were originally interpreted in terms of a spin-state equilibrium (Ehrenberg and Kamen, 1965). However, very recently it has been proposed (Maltempo et al., 1974; Maltempo, 1974; Maltempo and Moss, 1976) that the ground state consists instead of a novel admixture of high- and intermediate-spin ($S = 5/2$ and $3/2$) components not encountered previously. Oxidized cytochromes *c'* also exhibit complex behavior with pH variation (Taniguchi and Kamen, 1963; Horio and Kamen, 1961). In a recent detailed EPR study (Maltempo et al., 1974; Maltempo, 1974, 1975; Maltempo and Moss, 1976), nine magnetically distinct species have been reported in the pH range 1–13.

We present here measurements of the magnetic circular dichroism (MCD) (Stephens, 1974, 1976) of cytochrome *c'* in the near-infrared spectral region. The near-IR electronic transitions of heme proteins and their derivatives are more sensitive to the ligation, oxidation state, and spin state of the heme iron than are the visible (α, β, and Soret) transitions (Smith and Williams, 1970). MCD both enables these transitions to be more easily detected and yields more information than absorption spectroscopy (Cheng et al., 1973; Stephens et al., 1976). Thus, for example, the MCD of the near-infrared transitions of high- and low-spin ferric hemoglobin derivatives are diagnostic of the ground-state spin state (Stephens et al., 1976). Our objective has been to examine the information content of near-IR MCD in the case of the various forms of cytochrome *c'*. Specifically, we have studied the *c'* cytochromes obtained from the photosynthetic bacteria *Rhodospirillum*

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; IR, infrared; MCD, magnetic circular dichroism.

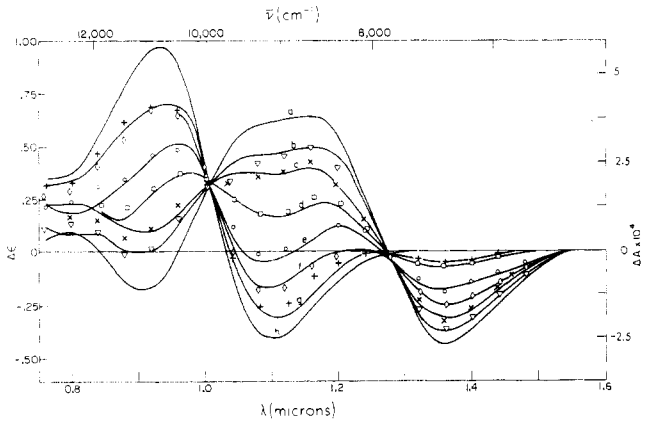

FIGURE 1: Near-IR MCD of oxidized *R. rubrum* cytochrome *c'* at pD: (a) 5.3, 6.2, 6.6, 7.1; (b) 7.7; (c) 8.1; (d) 8.4; (e) 8.8; (f) 9.0; (g) 9.4; (h) 10.0, 10.6, 11.0. $\Delta\epsilon$ and ΔA ($=(\Delta\epsilon)cl$) are normalized to H = +10 kG. ΔA values are for c = 0.66 mM, l = 1.0 cm. ▽, X, □, ○, ◇, and + indicate points calculated for pD values 7.7, 8.1, 8.4, 8.8, 9.0, and 9.4, respectively.

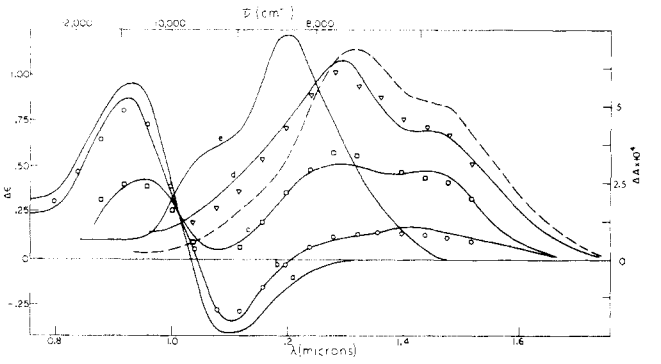

FIGURE 2: Near-IR MCD of oxidized *R. rubrum* cytochrome *c'* at pD: (a) 11.0, (b) 11.6, (c) 11.8, (d) 12.0, (e) 12.7. ΔA values are for c = 0.66 mM, l = 1.0 cm. The dashed curve is that calculated for species C. ○, □, and ◇ indicate points calculated for pD values 11.6, 11.8, and 12.0, respectively.

rubrum, Rhodopseudomonas palustris, and *Chromatium vinosum* at room temperature and, in the case of the oxidized proteins, as a function of pH. One of these, the *Rps. palustris* protein, is monomeric, while the other two are dimeric. The present work extends the earlier study of the near-IR absorption spectra of oxidized cytochrome *c'* in the pD range 6–13 (Kamen et al., 1973).

Experimental Procedures

Cytochromes *c'* from *R. rubrum, Rps. palustris,* and *C. vinosum* were preparations obtained as previously described (Bartsch, 1971). Human hemoglobin (two times crystallized), horse heart myoglobin, horse heart cytochrome *c* (type VI), all obtained from Sigma Chemical Co., were used as reference systems. Hemoglobin, myoglobin, and cytochrome *c* were oxidized with $Fe(CN)_6^{3-}$ and then dialyzed extensively.

The proteins were lyophilized and then dissolved in D_2O containing 0.01 M phosphate buffer (pD 7) for work in the near-IR. The pD was adjusted with solutions of NaOH or HCl in D_2O. Hemoglobin, myoglobin, and cytochrome *c* derivatives were made by addition of excess ligand. Myoglobin and *C. vinosum* cytochrome *c'* were reduced by addition of a slight excess of sodium dithionite to serum-capped cells maintained under a positive pressure of argon.

Absorption spectra were measured using a Cary 17. Near-IR CD and MCD were measured using an instrument described previously (Stephens et al., 1976, Osborne et al., 1973, Nafie et al., 1976). All measurements were made at room temperature. Absorption is reported in terms of absorbance A and molar extinction coefficient ϵ. CD is reported in terms of differential absorbance, ΔA, and differential molar extinction coefficient $\Delta\epsilon$. For MCD data, ΔA, and $\Delta\epsilon$ are normalized to a magnetic field of +10 kG. ϵ and $\Delta\epsilon$ values are per heme for comparison of monomeric and dimeric cytochromes. Protein concentrations were determined using visible ϵ values (Bartsch, 1971).

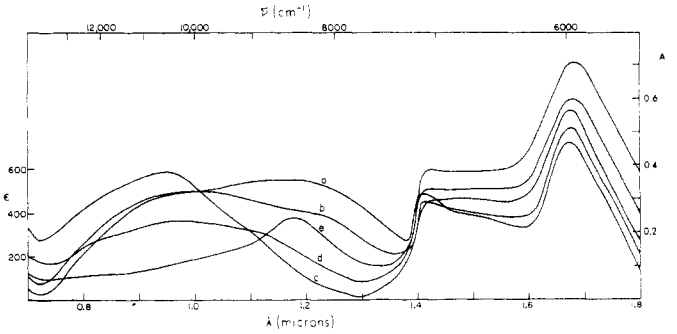

FIGURE 3: Near-IR absorption of oxidized *R. rubrum* cytochrome *c'* at pD: (a) 6.6, (b) 8.1, (c) 10.3, (d) 12.3, (e) 12.7. A values are for c = 0.66 mM, l = 1.0 cm.

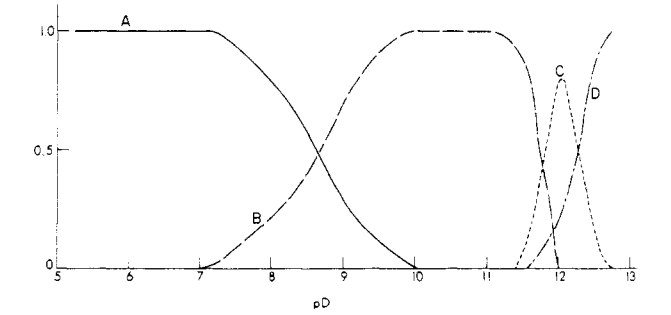

FIGURE 4: pD profiles of the mole fractions of species A, B, C, and D in oxidized *R. rubrum* cytochrome *c'*.

Results

The near-IR MCD and absorption spectra of oxidized *R. rubrum* cytochrome *c'* in the pD range 5–13 are shown in Figures 1–3. The absorption spectra arise both from heme electronic transitions and vibrational overtone transitions of protein and solvent. However, only the former transitions contribute observably to the MCD. The MCD in the pD range 5–11 exhibits isosbestic points, indicating the presence of two species A and B. Assuming the MCD of A and B to be those observed in the pD ranges 5.3–7.1 and 10.0–11.0, the MCD at intermediate pD values can be synthesised, as shown in Figure 1. The pD dependence of the mole fractions of A and B is shown in Figure 4. The midpoint of the transition occurs at pD 8.5. Above pD 11.0 further species appear. In the pD range 11.0–12.7, the MCD can be reproduced assuming three distinct species B, C and D, to exist, as shown in Figures 2 and 4. Species B and D are present at pD 11.0 and 12.7, respectively; species C is not present alone at any pD.

The pD dependence of the MCD and absorption of oxidized *Rps. palustris* and *C. vinosum* cytochromes *c'* is similar to that of *R. rubrum*. The midpoint of the A–B transition occurs at pD 7.8 and between 9 and 10 in the *Rps. palustris* and *C. vinosum* proteins, respectively. In the latter, the B–C transition

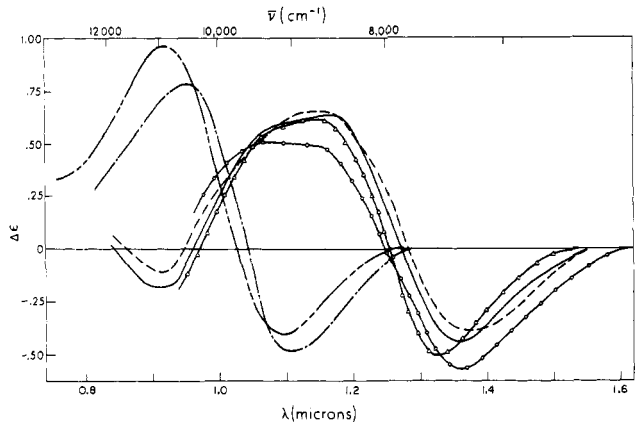

FIGURE 5: Near-IR MCD of oxidized cytochrome c': (—) *R. rubrum* (species A); (▽) *C. vinosum* (species A); (- - -) *Rps. palustris* (species A); (- - - -) *R. rubrum* (species B); (– – –) *Rps. palustris* (species B). ○ is the MCD of *R. rubrum* cytochrome c' at pD 1.1.

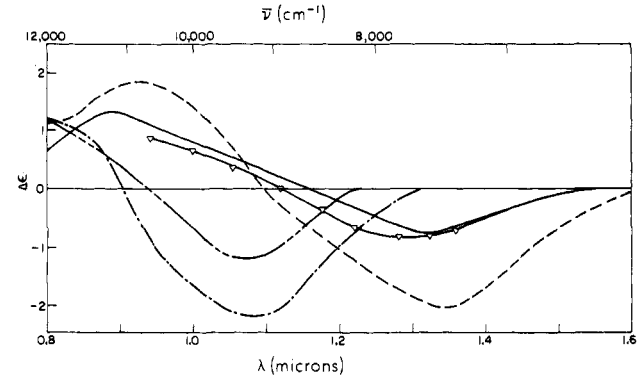

FIGURE 6: Near-IR CD of oxidized cytochrome c': (—) *R. rubrum* (species A); (△) *C. vinosum* (species A); (- - -) *Rps. palustris* (species A); (- - - -) *R. rubrum* (species B); (– – –) *Rps. palustris* (species B).

overlaps with the A–B transition and the midpoint is less easily determined.

The MCD of species A and B in the three cytochromes c' examined is compared in Figure 5. The variations among proteins are small. The MCD of *R. rubrum* cytochrome c' at pD 1.1 is also shown in Figure 5; the change in MCD from pD 7 is very small.

In addition to MCD, natural CD is also observed in the near-IR transitions of oxidized cytochromes c'. The CD of species A and B is illustrated in Figure 6. While the qualitative form does not depend on the protein, the magnitudes vary appreciably, the monomeric *Rps. palustris* protein exhibiting the largest CD.

The CD and MCD of reduced *C. vinosum* cytochrome c' at neutral pD are shown in Figure 7.

Figures 7–9 exhibit the near-IR MCD spectra of a number of heme protein derivatives which add to those reported earlier (Cheng et al., 1973, Stephens et al., 1976).

Discussion

The study of the near-IR electronic transitions of cytochrome c' by absorption spectroscopy is hindered by the weakness of the transitions (necessitating very concentrated solutions) and by overlapping vibrational absorption due to the protein, solvent, and buffer. MCD and CD are relatively insensitive to vibrational transitions (Nafie et al., 1976) and detect only electronic transitions at the sensitivity levels employed. These dichroic techniques thus possess a clear advantage in the detection of the near-IR electronic transitions, as illustrated by Figures 1–3. The bands observed in the absorption spectrum at $\lambda \gtrsim 1.4$ μm occur commonly in nonheme proteins (Stephens et al., unpublished work) and result from residual H_2O. These bands were also observed in an earlier near-IR study (Kamen et al., 1973) and incorrectly attributed to electronic transitions.

The number of species occurring over the pH range 1–13 detected by near-IR MCD in oxidized cytochrome c' can be compared with that identified by visible absorption spectroscopy and by EPR. Visible absorption studies have identified

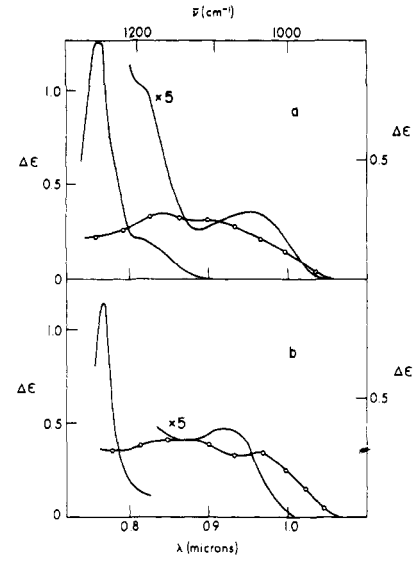

FIGURE 7: Near-IR CD (○) (right-hand scale) and MCD (—) (left-hand scale) of (a) reduced cytochrome c' and (b) reduced myoglobin at pD 7.0.

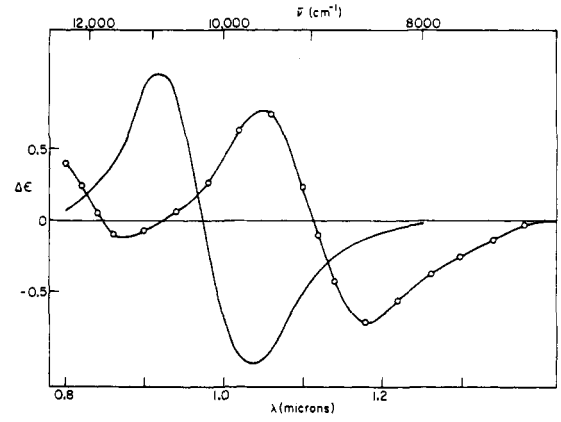

FIGURE 8: Near-IR MCD of oxidized hemoglobin derivatives: (—) formate; (○) acetate; both at pD 7.0.

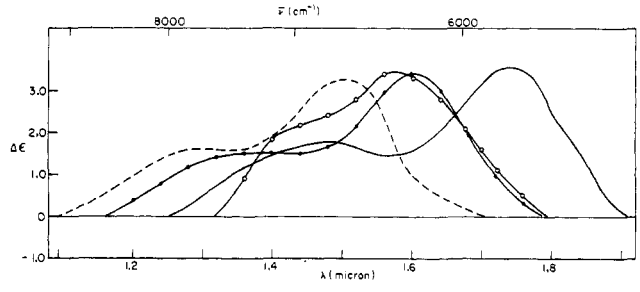

FIGURE 9: Near-IR mcd of: (—) oxidized cytochrome c; (●) imidazole derivative of oxidized hemoglobin; (○) lysine derivative of oxidized hemoglobin; (- - -) imidazole derivative of oxidized cytochrome c; all at pD 7.0.

three species on increasing pH from 7 to 13, an initial transition to a high-spin spectrum being followed by a transition to a low-spin (hemichrome) spectrum (Horio and Kamen, 1961; Imai et al., 1969). In contrast, low-temperature EPR studies (Maltempo et al., 1974; Maltempo, 1974, 1975; Maltempo and Moss, 1977 have yielded evidence for nine distinct species. At pH 7 two species, A_1 and A_2, are distinguished. At pH 11 a species B_2 occurs. Above pH 11.7 five species, B_2', B_3, C_1, C_2, and C_3, are identified. At pH 1, a further species B_1 is found. The near-IR MCD thus substantiates and extends the conclusions reached earlier by absorption spectroscopy and does not confirm the much larger number of species found by EPR. One may assume that the EPR species A_1 and B_2 correspond to species A and B, respectively, and that two of the species detected above pH 11 correspond to species C and D. The remaining species either differ too little in their room-temperature near-IR MCD to be distinguishable or are additional artifactual species created by the cooling of the protein to cryogenic temperatures.

In earlier studies of the near-IR MCD of ferric heme proteins (Cheng et al., 1973; Stephens et al., 1976), it was found that high- ($S = 5/2$) and low- ($S = 1/2$) spin hemes give rise to characteristic and radically different MCD spectra. Comparison with those earlier data shows that the MCD of species A and B are of high-spin form, while C and D exhibit low-spin spectra. The identification of species B as high-spin is consistent with the earlier optical, magnetic susceptibility and EPR work, taking B to be B_2 in the latter case. The assignment of C and D as low-spin is also consistent with earlier work, if we assume that C and D are two of the EPR species C_1, C_2, and C_3. Species A was first considered to be high-spin or to be in a high–low spin equilibrium. In recent EPR and low-temperature susceptibility studies (Maltempo et al., 1974; Maltempo, 1974, 1975; Maltempo and Moss, 1977), it was concluded, however, that at neutral pH oxidized cytochrome *c'* existed in two forms (A_1 and A_2), both of which possessed ground states consisting of intimately mixed high- and intermediate- ($S = 3/2$) spin forms. Furthermore, not only was the relative ordering of the $S = 5/2$ and $S = 3/2$ states opposite in A_1 and A_2, but a reversal in order was also claimed between the majority species (A_1) in *c. vinosum* and *R. rubrum* cytochromes. The MCD cannot categorically distinguish between a normal $S = 5/2$ and a mixed $S = 5/2, 3/2$ ground state at the present time because no reference data exist for definitively characterized mixed-spin species. However, the MCD does not appear to be anomalous for a high-spin species and gives no positive evidence for a mixed-spin species. In addition, since the MCD is sensitively related to the ground-state magnetic properties, one would expect a major change in MCD between proteins with opposite $S = 3/2, 5/2$ ordering. The absence of any significant difference in MCD among the three proteins examined (which include *C. vinosum* and *R. rubrum*) would appear to conflict with the EPR analysis. Thus, in sum, the MCD not only does not substantiate, but appears to be inconsistent with the details of the analysis of the neutral pH species in terms of a mixed high- and intermediate-spin state.

In addition to spin-state information, the energies of the near-IR bands of heme proteins are sensitive to, and, in principle, provide a means of elucidating, the axial ligation of the heme. The similarity in absorption, CD and MCD between reduced cytochrome *c'* and deoxymyoglobin (Figure 7) provides very strong evidence for identical ligation in these systems. If we then assume that the histidine ligand in reduced cytochrome *c'* remains bound in the oxidized proteins, the uncertainty in the latter reduces to the nature of the sixth ligands. Suitable model systems are provided by derivatives of oxidized myoglobin, hemoglobin, and cytochrome *c*. Consider first the high-spin species B. The near-IR band lies in the range found in the O-ligated systems Hb(H_2O) (Stephens et al., 1976), Mb(HCOO), and Mb(COOMe) (Figure 8). The possibility of coordination by H_2O can be eliminated because of the high pH at which species B occurs; in addition, the band energy in B is significantly higher than in Hb(H_2O). OH- and COOH-containing residues would thus appear to be the most likely candidates. Band energies are not known for OH-coordinated species. The closest analogue is HbOH, whose high-spin band (Stephens et al., 1976) lies appreciably higher in energy than that of species B. Ligation via a carboxyl group would thus appear more probable. It is also possible that the sixth coordination site is vacant. This appears unlikely on chemical grounds, but, since no near-IR data exist for species of this type, the possibility cannot be rigorously excluded. Coordination via nitrogen and methionine sulfur [cytochromes *c'* sequenced so far (Ambler, 1973; Meyer et al., 1975; Ambler et al., in preparation; it is worth noting that the sequences available thus far contain an invariant histidine adjacent to one heme-binding cysteine, providing further support for the assignment of histidine as a constant axial ligand of cytochrome *c'*) contain only the two cysteine residues binding the heme edge] is not expected to lead to a high-spin configuration. Of the various alternatives, we therefore tend toward the assignment of the sixth ligand as a carboxyl-containing residue. This possibility has been raised previously but on less certain grounds (Meyer et al., 1975). All cytochromes *c'* analyzed so far contain sufficient aspartate and glutamate residues to make carboxyl ligation plausible. Simple inspection of the primary structures for the three cytochromes *c'* studied as well as two others available (Ambler, 1973; Meyer et al., 1975; Ambler et al., in preparation) reveals no such residues present invariably in a fixed position in all five sequences. However, this circumstance obviously does not preclude substitution of carboxyl side-chain residues from positions not strictly homologous to each other. There are some instances found in these sequences wherein an aspartyl residue or glutamyl residue conserved in all but one or two chains can be imagined to be substituted by others not far removed along the chain. It is also interesting that a serine–threonine conserved position occurs at least once.

In the case of the neutral species A, the present unavailability of any model for the unusual properties exhibited precludes any definite statement regarding the axial ligand. However, since it is clear that the system is in the region where high- and low-spin configurations are close in energy, the ligand field strength of the sixth ligand is restricted. Since coordination through nitrogen or through methionine sulfur is expected to lead to a low-spin configuration and a vacant site should generate a high-spin state, these alternatives are unlikely, and again coordination via oxygen would seem indicated. In this case, the residue binding to the heme is either different from that in species B, or is the same with the pH change leading to a structural change adequately large to cause a significant perturbation in electronic structure. A possibility in the latter category is that the binding of a carboxyl group is modified as a result of the ionization of the lysine residue invariantly located adjacent to one cysteine residue (Ambler, 1973; Meyer et al., 1975; Ambler et al., in preparation), which is expected to occur around pH 9–10.

Turning to the high-pH, low-spin species C and D, comparison of Figure 2 with 8 shows that the near-IR bands of both

C and D occur at significantly higher energies than those of the models. As a result, coordination by methionine sulfur can be definitely excluded and lysine and histidine would also appear unlikely, particularly for species D. The possibility of binding by OH^- can also be eliminated by comparison with HbOH (Stephens et al., 1976). This would seem to leave OH-bearing residues as the most likely remaining alternative. However, we have no data on adequate models for OR^--coordinated hemes so that such an assignment is speculative.

Conclusion

We have demonstrated, first, that the location of the near-IR electronic transitions of cytochrome c' is more reliably effected using MCD than by absorption spectroscopy. Secondly, we have shown that the pH dependence of the near-IR MCD of oxidized cytochrome c' can be satisfactorily interpreted on the basis of four distinct species. The much larger number of species apparently detected by low-temperature EPR is not substantiated. Third, the MCD of the neutral pH species is not inconsistent with a high-spin ground state and provides no positive evidence in favor of the suggestion that a mixed-intermediate–high-spin state exists. Fourth, the near-IR band energies enable the range of possible axial ligands to be narrowed. Histidine is implicated as an axial ligand and oxygen-coordinated ligands appear to be the most likely candidates for the sixth ligand position in all four oxidized cytochrome c' species. Lastly, the near-IR MCD data provide a basis for the critical evaluation of future cytochrome c' model compounds.

Further work is required in two directions: first, the extension of the MCD measurements to low temperatures should enable the assignment of the ground states of the various species to me more definitively established and a direct link to the EPR data to be made. Second, the synthesis and near-IR study of potential model systems which are currently unavailable is urgently needed. Models for ferric hemes axially coordinated by histidine and by either carboxyl- or hydroxyl-bearing residues appear to be of prime importance. In our opinion, such work should permit the spin states and ligations of cytochrome c' to be definitely established.

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