Correlations between Structural and Spectroscopic Properties of the High-Spin Heme Protein Cytochrome c'[†]

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ABSTRACT: The cytochromes c' are a class of heme proteins whose native spectroscopic properties have been suggested to represent a quantum mechanical admixture of intermediate- ($S = {}^3/_2$) and high- ($S = {}^5/_2$) spin states. Here features of the cytochrome c' heme environment, as revealed by X-ray crystallographic studies of the dimeric cytochrome c' from *Rhodospirillum molischianum*, are related to the observed spectroscopic properties. The environment of the heme group in cytochrome c' supports the existence of the admixed spin state at neutral pH and suggests that pH-dependent transition to a pure high-spin state at alkaline pH involves deprotonation of the histidine axial ligand to the heme iron.

The majority of known heme proteins show spectroscopic properties that characterize their iron electronic states as either pure low- ($S = {}^1/_2$) or high- ($S = {}^5/_2$) spin species. Nevertheless, a number of heme proteins having anomalous properties are known. Among these are the bacterial cytochromes c' whose spectroscopic behavior has been suggested to reflect a quantum mechanical admixture of intermediate- ($S = {}^3/_2$) and high- ($S = {}^5/_2$) spin states (Maltempo, 1974; Maltempo & Moss, 1976; Maltempo et al., 1974). Recently, the tertiary structure of *Rhodospirillum molischianum* ferricytochrome c' has been determined at 2.5-Å resolution (Weber et al., 1980, 1981). The present paper describes features of the heme environment and axial ligation pattern in cytochrome c' that may give rise to its unusual spectroscopic properties.

Discussion

Quantum Mechanical Admixture of Intermediate- and High-Spin States. A variety of experimental observations suggest an unusual spin state in cytochrome c'. The magnetic susceptibility and EPR properties of the ferricytochrome c' heme iron are intermediate between those of pure low- or high-spin heme proteins (Ehrenberg & Kamen, 1965; Tasaki et al., 1967). Nevertheless, resonance raman data suggest that the electronic configuration of the heme iron represents a magnetically pure state and the heme geometry differs from that typically observed in pure high-spin heme proteins (Strekas & Spiro, 1974; Kitagawa et al., 1977; Spiro et al., 1979). Maltempo accounted for these observations by suggesting that the electronic distribution of iron d orbitals in ferricytochrome c' represented a quantum mechanical admixture of intermediate- ($S = {}^3/_2$) and high- ($S = {}^5/_2$) spin states (Maltempo, 1974; Maltempo et al., 1974).

Figure 1 diagrammatically shows the relationship between axial ligand field strength and spin state in ferric porphyrins. Low-spin ($S = {}^1/_2$) species have strong field axial ligation. Weaker field ligation results in an energy level distribution of iron d orbitals characteristic of the high- ($S = {}^5/_2$) spin state, whereas intermediate- ($S = {}^3/_2$) spin species exist only when the heme iron is axially ligated by a very weak field ligand. According to Maltempo (Maltempo, 1974; Maltempo et al., 1974; Maltempo & Moss, 1976), the electronic configuration of a ferric heme iron could include contributions from more than one of the pure spin states. The resultant quantum mechanical admixture would correspond to a single magnetic species. This admixture differs from a thermal mixture that can be described as the sum of magnetically distinguishable pure spin states. As discussed in the caption to Figure 1, the electronic distribution of the admixture differs from a pure high-spin state by increased occupation of orbitals in the heme plane and a corresponding decrease along the axial ligand direction. Proposed structural mainfestations included shortened iron-to-pyrole nitrogen distances and small iron displacement from the mean heme plane.

A pentacoordinate ferriporphyrin compound having perchlorate as a single weak field ligand has recently been synthesized (Dolphin et al., 1977; Kastner et al., 1978; Reed et al., 1979; Ogoshi et al., 1980; Goff & Shimomura, 1980). In the solid state, this compound has magnetic properties that suggest the existence of a quantum mechanical admixture of intermediate- ($S = {}^3/_2$) and high- ($S = {}^5/_2$) spin states (Reed et al., 1979; Goff & Shimomura, 1980; Ogoshi et al., 1980). X-ray crystallographic studies (Reed et al., 1979) of this molecule show a smaller iron displacement from the average plane of the porphyrin ring and shorter iron-to-porphyrin nitrogen distances compared to those typically observed in high-spin heme model complexes. Both the absence of a sixth iron ligand and a relatively long iron-to-perchlorate oxygen bond length are consistent with very weak field ligation to the porphyrin iron. The structural properties of this molecule therefore appear to be consistent with the theoretical expectations for a quantum mechanical admixture in ferric porphyrins (Maltempo, 1974; Reed et al., 1979).

Cytochrome c' Heme Environment. Figure 2 shows the orientation of amino acid side chains near the heme group in *R. molischianum* cytochrome c'. Structural comparisons of ferricytochrome c' with the high-spin heme proteins cytochrome c peroxidase (Poulos et al., 1980), the globins (Perutz, 1979), and catalase (Murthy et al., 1981) show critical differences in heme environment and iron axial ligation. In particular, only in cytochrome c' is the ferriheme iron pentacoordinate, with the histidyl axial ligand exposed to solvent. In other high-spin heme proteins, the Nδ1 nitrogen of the histidine axial ligand is hydrogen bonded to a protein oxygen atom (Salemme et al., 1973; Valentine et al., 1979; Poulos & Kraut, 1980). This is relevant because hydrogen bonding to

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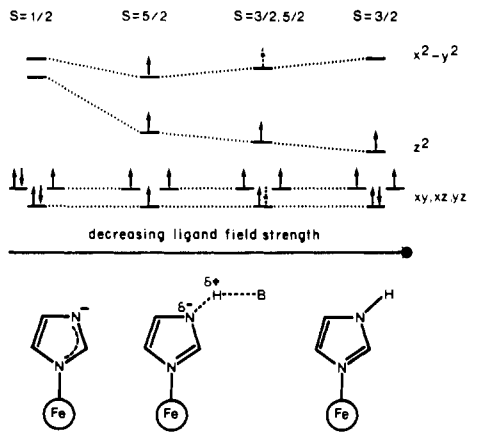

FIGURE 1: Correlations between spin state and ligand field strength in ferric porphyrins. The arrow in the center of the figure indicates the direction of decreasing ligand field strength. The upper part shows the electronic distribution of iron d orbitals in the low- ($S = {}^1/_2$), high- ($S = {}^5/_2$), admixed ($S = {}^3/_2$, $S = {}^5/_2$), and intermediate- ($S = {}^3/_2$) spin states. The lower part shows the relationship between the protonation state of an imidazole axial ligand and its ligand field strength. The upper part is according to Reed et al. (1979); the lower part is according to Landrum et al. (1980). As shown in the upper part of the figure, the low- ($S = {}^1/_2$) and high- ($S = {}^5/_2$) spin states differ in the energy separation of the $d_{x^2-y^2}$ and d_{z^2} orbitals from the lower lying d_{xy}, d_{xz}, and d_{yz} orbitals. The intermediate- ($S = {}^3/_2$) spin state arises when the difference in energy between $d_{x^2-y^2}$ and d_{z^2} orbitals is greater than the energy difference between the d_{z^2} and d_{xz}, d_{yz}, and d_{xy} orbitals. In this situation, the $d_{x^2-y^2}$ orbital remains unoccupied and the d_{xy} orbital contains a pair of electrons. Orbital selection rules show that the intermediate- ($S = {}^3/_2$) spin state can mix with either the low- or high-spin configurations. Mixing can occur if the energy separation between spin states is comparable to the spin orbit coupling constant. The quantum mechanical admixture of intermediate- and high-spin states arises when the antibonding $d_{x^2-y^2}$ orbital is raised sufficiently in energy so that electrons normally occupying this orbital in the pure high-spin state would partially occupy both the $d_{x^2-y^2}$ and d_{xy} orbitals. In porphyrin systems, lobes of the d_{xy} and $d_{x^2-y^2}$ orbitals all lie in the porphyrin plane, while the d_{yz}, d_{xz}, and d_{z^2} orbitals are above and below the plane. For this reason, the spin state is sensitive to the ligand field strength of the iron axial ligands. For either the intermediate-spin or the admixed-spin state, weak field ligation to the heme iron is required in order to keep the d_{z^2}, d_{xy}, d_{yz}, and d_{xz} orbitals close in energy. The lower part of the figure shows the correspondence between protonation state and ligand field strength for imidazole axially ligated to iron. Strongest ligand field behavior is exhibited when the Nδ1 is fully deprotonated (Landrum et al., 1980), as in the imidazolate form shown at the extreme lower left. When Nδ1 is fully protonated, weakest field ligation by this side chain is observed. The middle figure shows the sitation most frequently observed in heme protein structures. The histidine axial ligand is hydrogen bonded to a basic atom of the protein. Hydrogen bonding to Nδ1 results in a partial charge on the imidazole ring. The ligand field strength of this hydrogen-bonded imidazole is between that of the imidazolate (left) and that of fully protonated (right) forms.

Nδ1 is thought to increase the imidazole ligand field strength (Peisach, 1975; Nappa et al., 1977; Valentine et al., 1979). Conversely, the lack of a hydrogen bond to Nδ1 of the coordinate imidazole suggests that histidine behaves as a weaker field ligand in cytochrome *c′* than in other high-spin heme proteins. This and the absence of a sixth axial ligand to the heme iron (Emptage et al., 1981; Weber et al., 1981) indicate an overall weak field axial coordination to the cytochrome *c′* heme iron.

While detailed structural comparisons of the intermediate-spin model porphyrin and cytochrome *c′* heme group await completion of the protein's refinement, current 2.5-Å resolution maps show the heme iron apparently closer to the porphyrin plane than in comparable resolution maps of the high-spin ferriheme protein cytochrome *c* peroxidase (Poulos et al., 1980). Therefore, weak field iron ligation and the small iron displacement from the porphyrin plane appear to be common structural features consistent with an admixed-spin state in both the porphyrin model complex and cytochrome *c′*. Additional evidence for existence of the admixed spin state is suggested by the pH-dependent spectral properties of ferricytochrome *c′*.

Spectral Transitions in Ferricytochrome c′. Cytochrome *c′* exhibits at least two spectroscopically distinguishable high-spin states as a function of pH. The proposed admixed state has been associated with the spectra observed at neutral pH (state I). As the pH is increased, another high-spin species having a distinct optical spectra (state II) is observed (Horio & Kamen, 1961). The pH-induced spectral transition, whose p*K* ranges from 7.1 to 9.0 among various species of cytochrome *c′* (Bartsch, 1978), is accomplished with retention of the native protein structure (Imai et al., 1969a,b; Emptage et al., 1981).

A variety of experimental results suggests that the pH-induced spectral transition is associated with a shift to a high-spin heme electronic species. These include MCD (Rawlings et al., 1977) and Mossbauer and EPR mesaurements (Ehrenberg & Kamen, 1965; Emptage et al., 1977), which indicate that the iron configuration in state II is pure $S = {}^5/_2$. These experiments also show, by decreases in both quadrupole splitting and the zero field splitting parameter, that the distribution of electrons about the iron nucleus is more symmetric at higher pH. Among the pure spin states, only the asymmetric electronic distribution in the $S = {}^3/_2$ spin state results in a quadrupole splitting. Consequently, a decrease in quadrupole splitting in the state I to II transition suggests a change from an admixed to a pure high-spin electronic state. Further evidence comes from resonance raman data (Strekas & Spiro, 1974; Kitagawa et al., 1977; Spiro et al., 1979). These show the heme iron further displaced from the porphyrin plane in state II, an observation consistent with a transition from an admixed spin state (where the heme iron is slightly displaced from the heme plane) to a pure high-spin state having greater iron displacement.

Transition from an admixed spin state to a pure high- ($S = {}^5/_2$) spin state would require either an increase in the field strength of the axial ligand and/or additional ligation at the sixth axial site. Given that the state I to II transition in cytochrome *c′* is accomplished without structural rearrangement (Imai et al., 1969a,b; Emptage et al., 1981) and that the imidazole is a stronger field ligand when deprotonated (Landrum et al., 1980), the spectral transition is most likely to reflect deprotonation of Nδ1 of the histidine axial ligand.

Evidence for this proposal comes from a variety of sources. Although the p*K* for the imidazole Nδ1 proton is 14.4 in water (Yagil, 1967), it is lowered in histidine transition metal complexes because the negative charge formed on deprotonation can be partially delocalized to the positively charged metal. In pentaammineruthenium histidine, for example, the Nδ1 p*K* is 8.8 (Sundberg & Gupta, 1973). Acidification of Nδ1 has also been observed in high-spin, six-coordinate ferric heme proteins, *Chironomus* methemoglobin and sperm whale myoglobin, where the Nδ1 p*K*s range from 9.0 to 10.4 (Mohr et al., 1967; Morishima et al., 1980). However, in the metglobins, the effective charge on the hexacoordinate ferriheme iron is additionally delocalized to an electronegative oxygen atom at the sixth axial position. Therefore, absence of an electronegative distal ligand in cytochrome *c′* might be expected to additionally acidify the Nδ1 proton to within the pH region of the observed spectral transition.

It has been suggested that the state I to II transition is caused by an oxygen-containing side chain that either binds

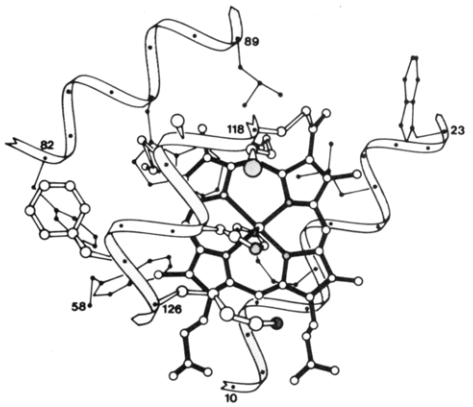

FIGURE 2: Schematic representation of the heme environment in *R. molischianum* cytochrome *c'*. The view is approximately parallel to the iron–axial ligand bond, looking from the solvent toward the molecular interior. Ribbons indicate segments of α helix with α-carbon positions dotted. Nitrogen atoms are shaded. The Nδ1 nitrogen of His-122 is closest to the viewer. Hydrophobic residues, Met-16 and Leu-19, and three aromatic residues, Trp-58, Phe-75, and Phe-95, are located near the interior heme face. Tryptophan, Trp-23, and phenylalanine, Phe-125, side chains pack along the heme edge and are slightly exposed to solvent. In contrast to the close packing of residues about the interior face and sides of the porphyrin plane, the opposite heme face is partially exposed to solvent. Other than the heme-binding sequence, Cys-118, Lys-119, Ala-120, Cys-121, and His-122, only one other residue, Lys-126, packs near the exterior heme face. The heme iron is ligated by four pyrole nitrogens of the porphyrin ring. The imidazole Nϵ2 of His-122 provides the fifth axial ligand to the heme iron. The sixth axial ligation site is unoccupied.

to the sixth axial position of the heme iron at alkaline pH or whose deprotonation in the vicinity of the heme group is responsible for the observed spectral change (Rawlings et al., 1977; Emptage et al., 1981; La Mar et al., 1981). In the case of *R. molischianum* cytochrome *c'*, no oxygen-containing side chains are located within the distal heme pocket (Figure 2). Coordination by a side chain or backbone oxygen would require considerable reorganization of the polypeptide, in contradiction of existing experimental evidence that indicates that no significant conformational changes in the polypeptide backbone occur (Imai et al., 1969a,b; Emptage et al., 1981). The second proposal, that ionization of an oxygen-containing residue located near the carboxy terminus induces the heme spectral transition, appears equally unlikely. Although aspartate and glutamate residues are sequentially located near the carboxy terminus, these side chains in *R. molischianum* cytochrome *c'* are further than 10 Å from the heme group. Moreover, they are exposed to solvent, and so would have expected p*K*s near their solution values.

Conclusion

The structure determination of *R. molischianum* cytochrome *c'* shows the heme iron to be pentacoordinate with a solvent-exposed histidine side chain as the single axial ligand. The axial ligation pattern suggests weaker field ligand interactions than observed in the structures of other high-spin heme proteins. Weak field axial ligation and a iron position near the porphyrin plane are structural features consistent with proposals that the electronic configuration of the heme iron in cytochrome *c'* represents a quantum mechanical admixture of intermediate- ($S = {}^3/_2$) and high- ($S = {}^5/_2$) spin states. This suggestion is also supported by data that indicate that at higher pH, a stronger axial ligation pattern results in a pure ($S = {}^5/_2$) high-spin species. The arguments presented here are consistent with recent experimental data on the pH-dependent NMR spectra of ferricytochrome *c'* (J. T. Jackson and G. N. LaMar, unpublished results) that indicate that the pH-induced spectral transition between high-spin states is linked to the protonation state of the heme iron histidine axial ligand.

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Proton Nuclear Magnetic Resonance Spectra of *Pseudomonas aeruginosa* Ferricytochrome cd_1†

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ABSTRACT: Proton nuclear magnetic resonance spectra of ferricytochrome cd_1 from the denitrifying bacterium *Pseudomonas aeruginosa* have been obtained. The normal 0–10-ppm chemical shift range shows many overlapping and nonresolvable peaks, as would be expected for a dimeric protein of molecular weight approximately 120 000. In the downfield region between 10 and 50 ppm, and in the upfield region between 0 and −20 ppm, resolvable resonances corresponding to a small number of protons are observed. The temperature and pH behavior of these resonances have been examined. For some of the resolved resonances, the pH behavior of chemical shifts and intensities indicates that the oxidized form of the enzyme undergoes a structural transition with a pK of 5.8 ± 0.3. On the basis of several lines of evidence, some assignments are proposed in which resolvable resonances are assigned as originating from either the heme c or the heme d_1 prosthetic groups of the enzyme.

Cytochrome cd_1 is a dissimilatory nitrite reductase found in facultative denitrifying bacteria. In its oxidation–reduction cycle, it accepts reducing equivalents from donor cytochromes c and transfers these to nitrite, reducing it to nitric oxide predominantly (Wharton & Wintraub, 1980). In a nonphysiological reaction, it also may reduce oxygen to water (Timkovich & Robinson, 1979). The native enzyme is a dimer composed of two identical subunits. Each subunit has a molecular weight of approximately 60 000 and contains one covalently bonded heme c and one noncovalently bonded heme d_1 (Kuronen et al., 1975). The structure and characteristics of this enzyme, especially as isolated from *Pseudomonas aeruginosa*, have been extensively explored by a variety of spectroscopic techniques [see Cotton et al. (1981) and references cited therein]. The purpose of this report is to present the application of proton nuclear magnetic resonance (NMR) to structural studies of *Pseudomonas* cytochrome cd_1.

Proton NMR has yielded important structural information on heme proteins, most notably on the environment of the heme prosthetic groups. For cytochrome cd_1, the application of NMR presents formidable problems arising from the large molecular weight of the native enzyme. The spectral region between 0 and 10 ppm has so many overlapping resonances from the amino acid residues that no useful spectroscopic information has been obtained to date. However, hyperfine chemical shifts from the heme iron are expected to place some resonances outside of the main protein envelope. Although there are four hemes per native unit, the NMR line widths are influenced by paramagnetic broadening as well as by the correlation time for the enzyme as a whole. This correlation time corresponds to a molecular weight unit of 120 000. Very broad resonances are therefore expected and have been observed in the ensuing spectra. Nevertheless, the hyperfine-shifted resonances cover a large shift range and are extremely sensitive to heme environment so that useful structural and mechanistic information is obtainable.

Materials and Methods

Pseudomonas aeruginosa (ATCC 19429) was cultured in 501 lots, and cytochrome cd_1 was purified as described by Parr et al. (1976) with the following exception. The first ammonium sulfate fraction precipitating between 40 and 90% saturation from the crude extract was redissolved in pH 7.5 phosphate buffer and applied to a gel permeation column (5.0 × 90 cm) of Sephacryl S-200 (Pharmacia Fine Chemicals). On the basis of molecular weight, the green cytochrome cd_1 was well resolved from cytochrome c-551 and azurin in this step. Thereafter, ion-exchange chromatographic steps were performed as described by Parr et al. (1976). The final material was typically ≥95% pure as judged by the Soret to 280-nm spectroscopic ratio index. In some initial experiments, protein only 80% pure was used. At this level, the cytochrome has been purified from other heme proteins, and the spectral regions outside the 0–10-ppm range are indistinguishable from those for pure protein. Protein was stored frozen in liquid nitrogen or as a suspension in 80% saturated ammonium sulfate at 4 °C. Protein was concentrated for NMR studies by the following procedure. Solutions were brought to 80%

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