Proton Magnetic Resonance Evidence for Methionine-Iron Coordination in Mammalian-type Ferrocytochrome c

by

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Summary

Proton magnetic resonance spectra of mammalian-type ferrocytochromes c from nine species ranging from yeast to mammals provide evidence for identical methionine-iron coordination in all these protein molecules.

The iron atom of the heme prosthetic group of cytochrome c has six coordinate binding sites, four of which are occupied by the four planar nitrogen atoms of the porphyrin ring. The protein polypeptide chain is coordinated to ligand positions 5 and 6 of the heme iron through functions of two amino acid residues. In spite of considerable research, rigorous identification of these residues has proved difficult and, indeed, some investigators have suggested that the ligands may not be the same in ferri- and ferrocytochrome c. Considerable evidence has been obtained that histidine-18 (mammalian-type cytochrome c) is bound to iron at the 5th ligand position, and other histidine and lysine residues have been suggested as prob-

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able ligands at the 6th position. The unresolved state of this problem was authoritatively reviewed by Margoliash and Schejter in 1966.[1] Subsequently, a number of investigators[2-8] have provided indirect evidence that the sulfur of the methionine-80 residue (mammalian-type cytochrome c) is bound at the 6th ligand position to the heme iron in both ferri- and ferrocytochrome c. (The corresponding ligands are histidine-16 and methionine-61 in Pseudomonas cytochrome c[3].) X-ray studies of horse ferricytochrome c by Dickerson and his colleagues[9-11] confirmed the binding of the imidazole ring of histidine-18 to the 5th ligand position of the heme iron. The X-ray results were also compatible with binding of methionine-80 at the 6th ligand position but could not rule out the possibility that this ligand might be a lysyl side chain. We have examined the proton magnetic resonance (PMR) spectra of mammalian-type ferrocytochromes c from nine species and present here evidence for binding of methionine to the heme-iron in each of these ferrocytochromes c.

PMR spectra of the ferrocytochromes c were obtained with a Varian Associates 220 MHz spectrometer from solutions of the proteins (10%) in D_2O at 40°C, pD between 6.0 and 7.5. The solutions were prepared from the corresponding ferricytochromes c by reduction with ascorbic acid. Resonance positions are referred to the position of the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) used as an internal reference; negative shifts are to high field. The signal-to-noise ratio was improved by use of a Varian Associates C-1024 computer of average transients.

Two of the cytochromes c were commercial materials, horse (Sigma Type III) and yeast, Candida krusei (Calbiochem A grade), and were examined without further purification. Purified cytochromes c from turkey, duck, hog, and pigeon were kindly provided by Drs. R. W. Estabrook and A. Kowalsky. Bovine, chicken and tuna cytochromes c were prepared and purified by the method

of Margoliash and Walasek.[12]

PMR spectra of the nine ferrocytochromes c for the region of resonance absorption from about 0 to -4.0 ppm are shown in Figure 1. These ferrocytochromes c exhibit, in most instances, small differences in their amino acid compositions and their PMR spectra are different in other spectral regions. The notable feature however in the spectral region shown in Figure 1 is the constancy of the spectra from the various

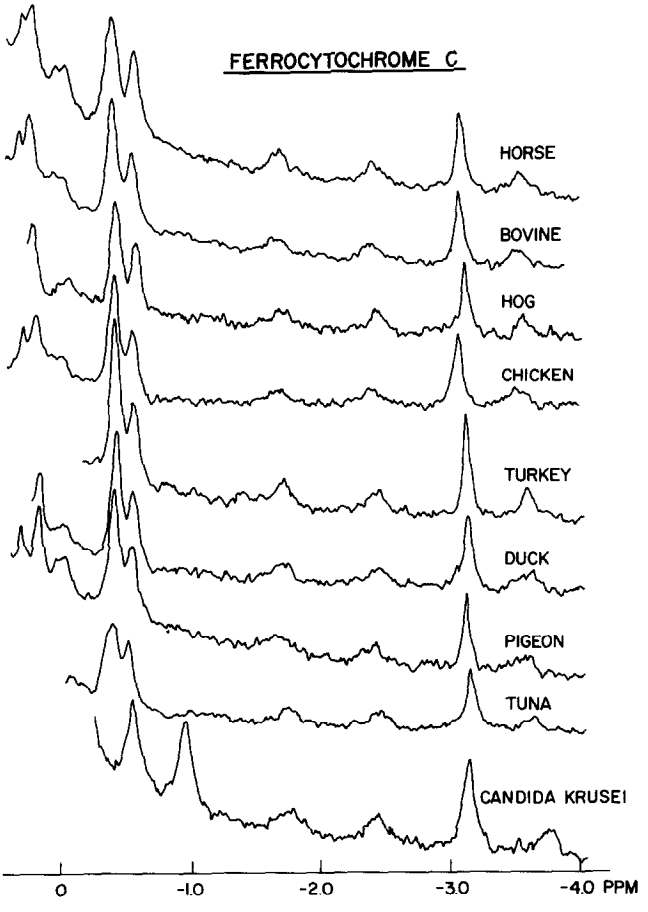

Fig. 1. 220 MHz PMR spectra from 0 to -4.0 ppm of ferrocytochromes c in D_2O, pD 6.0 to 7.5 at 40°C. Reference, internal DSS; negative shifts to high field.

species. The resonances at about -1.75, -2.45, and -3.55 ppm correspond in intensity to single protons; the resonance at -3.15 corresponds to three protons. The latter resonance is assigned to protons of a freely rotating methyl group (therefore having three equivalent hydrogen atoms) since it has not provided any evidence of arising from fortuitous overlap of non-equivalent protons throughout studies of ferrocytochrome c with a wide variation of solution conditions. Similarly the resonance group at about -0.5 to -0.7 ppm is assigned to three methyl groups (except for Candida krusei), two centered near -0.5 and one at -0.7 ppm. These individual resonances are all clearly resolved at higher temperatures, as one of the resonances at -0.5 ppm moves to lower field as temperature is increased.

If the protons of the amino acid residues of a protein are not influenced by their environment in the protein molecule, their resonances all occur at field positions to low field from about +0.9 ppm.[13] That is, resonances are only observed in the spectral region of Fig. 1 for protons that are in particular shielding environments in the protein molecule that cause rather large high-field shifts. For a diamagnetic protein such as ferrocytochrome c, the only protons expected to exhibit resonances above 0 ppm are those in close juxtaposition to the faces of aromatic rings so that they experience ring-current magnetic fields from these conjugated cyclic structures that shift resonances to higher fields. These ring-current fields arise in ferrocytochrome c both from the aromatic amino acid residues and from the porphyrin ring of the prosthetic group. Estimates of the magnitudes of ring-current fields of amino acid residues and observations on PMR spectra of other proteins[14,15] indicate that even when a proton is surrounded by such residues the high-field shift is not likely to exceed 3 ppm and not likely, therefore, to produce resonances to high field of -2.0 ppm. However, it is well established that very large ring current fields can be

associated with porphyrin rings.[16,17] Therefore, we may conclude that the resonances in the -2.0 to -4.0 ppm spectral region arise from amino acid residue protons close to the face of the heme ring (resonances of protons of the prosthetic group occur far to low field). Aromatic residues may, of course, also contribute to the ring-current field that shifts these resonances. The strongest high-field shifting ring-current field of the porphyrin ring is along the symmetry axis perpendicular to the ring plane. Thus, resonances of protons of ligands of the heme-iron at the 5th and 6th ligand positions are expected to experience the largest high-field shifts and no other residues are likely to be in a sufficiently strong ring-current field to exhibit resonance in field regions above -2.0 ppm.

The unperturbed positions of the imidazole protons of histidine are too far to low field (7 to 8 ppm) for these protons of histidine-18 to be shifted into the high-field region shown in Fig. 1. The unperturbed resonance positions of the α and β histidine protons are to higher field (3 to 4 ppm), but these protons for histidine-18 are somewhat removed from the face of the porphyrin ring and the symmetry axis so they are also unlikely to be found in the -2.0 to -4.0 ppm field range. Thus, we conclude that the 3 resonances observed in the field region probably arise from the residue at the 6th ligand position of the heme-iron. These resonances can be accounted for very nicely if this residue is methionine bound via the sulfur atom to the heme-iron and indeed any other assignment seems improbable. First, one of the resonances arises from a methyl group. No residue type except methionine contains both a coordinating function group and a methyl group (unless coordination via the peptide nitrogen or carbonyl is invoked). Assignment of this resonance to a methionine methyl is also supported by the sharpness of the resonance peak. Methyl resonances of all other amino acid residues that contain methyl

groups have rather rounded intensity maxima since the observed resonances are envelopes of unresolved nuclear spin-spin fine structure; the methyl proton resonance of methionine does not exhibit resolvable nuclear spin-spin fine structure. If methionine is bound to the heme-iron via the methionine sulfur atom, both the methyl protons and γ protons are close to the heme-face and in a very strong high-field directing ring-current field. The equivalence of the methyl protons indicates free rotation of the methyl group. One does not expect the two γ protons, however, to be in identical environments since rotation of the β and γ methylene groups is sterically restricted. The single proton resonances at -2.45 and -3.55 are assigned to the methionine γ protons. The methionine β protons and α proton are farther from the heme face and therefore subject to smaller ring-current fields. The resonance at -1.75 is presumed to arise from one of those three protons; the resonances of the other two must occur in the incompletely resolved spectra to lower field.

Thus, the PMR spectra of nine mammalian-type ferrocytochromes c support the hypothesis that methionine is coordinated to the heme-iron of these molecules. Work is in progress to extend these observations to bacterial cytochromes c. For the ferricytochromes c, one expects resonances of protons of the heme-iron ligands to be greatly shifted from the positions for the ferrocytochromes c because of the paramagnetism of the iron atom. Wüthrich and Shulman[18] have recently identified a feature of PMR spectra of several mammalian-type ferricytochromes c that indicates that methionine-iron coordination is also a general feature of the oxidized state of cytochrome c.

We noted above that three methyl resonances in the field region from -0.5 to -0.7 ppm are a constant feature of all the ferrocytochromes c examined except Candida krusei. The shifts of these resonances to high field from the unperturbed

methyl region is not so great that they must lie close to the heme faces; a very favorable juxtaposition to aromatic residues would be sufficient. However, it is of some interest to determine from the preliminary X-ray information[9-10] whether groups close to the heme-faces could provide these resonances. We find that this is indeed the case; threonine-19 and isoleucine-81 appear to be the only methyl-containing residues (in addition to met-80) that are close enough to the heme-faces to experience a substantial high-field directing ring-current field. Threonine-19 occurs in all the ferrocytochromes c examined including Candida krusei and may account for one of the methyl resonances. Isoleucine-81 occurs in all the ferrocytochrome c species except Candida krusei where it is replaced by alanine. Thus, isoleucine-81 may provide two methyl resonances in slightly non-equivalent environments, that are replaced by one alanine methyl resonance for Candida krusei.

A report comparing other spectral regions of PMR spectra of cytochromes c from different species is in preparation.

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