A MÖSSBAUER STUDY OF FERRI- AND FERROCYTOCHROME *C*

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ABSTRACT The Mössbauer spectra of horse heart ferri- and ferrocytochrome *c* were obtained at room temperature using lyophilized powders. The Mössbauer data indicate that the iron in both lyophilized samples is in a low-spin state. The high quadrupole splittings suggest that the iron atom is in an asymmetric ligand field. Upon reduction the asymmetry increases, suggesting a change in the bonding between the protein moieties and the iron atom.

INTRODUCTION

Mössbauer spectroscopy can be used to measure the state and bonding of a metallic ion. The magnitude of the parameters, chemical shift and quadrupole splitting, can be related to the coordination of the metal with the other constituents of a biochemical. Of particular interest in studying biochemicals are those containing iron. The majority of previous studies with Mössbauer spectroscopy have been concerned with hemoproteins and heme compounds (1–8). In this study, the Mössbauer spectra of cytochrome *c* in both oxidation states are interpreted to assist in determining the symmetry and bonding of the ligands to the iron.

EXPERIMENTAL TECHNIQUES

Horse heart cytochrome *c* with a 0.43% iron content was purchased from Sigma Chemical Company. The reduced form was prepared from the ferricytochrome *c* by the addition of sodium dithionite (5 mg/ml) to a solution of cytochrome *c* (50 mg/ml) in 0.1 M phosphate buffer at pH 7.0. It was assumed that total reduction had taken place since no change occurred in the reduced absorption spectrum (recorded on a Beckman DK-1-A spectrophotometer) with the addition of an excess amount of dithionite to the cuvette.

The absorbers for the Mössbauer experiment contained 250 mg/cm^2 of the dry protein in a plastic cylinder mounted on a Plexiglas plate. The Mössbauer spectrometer was a Nuclear Science and Engineering Model B constant velocity advance. The source was 20 mc of ^{57}Co diffused into palladium supplied by New England Nuclear Corporation. The detector was a Reuter-Stokes proportional counter containing 10% methane and 90% xenon. The source

and the absorber were maintained at room temperature (298°K) throughout the measurements.

RESULTS AND DISCUSSION

The Mössbauer spectra of ferri- and ferrocytochrome *c* are shown in Fig. 1 a and b. The Mössbauer parameters obtained from these spectra are given in Table I. The quadrupole splitting of the ferricytochrome *c*, 0.190 cm/sec, is identical with that obtained by Maling and Weissbluth (2) and compares favorably with the value of 0.205 cm/sec obtained by Karger (3). Maling and Weissbluth (4) recently reported a quadrupole splitting of 0.13 cm/sec for ferrocytochrome *c*.

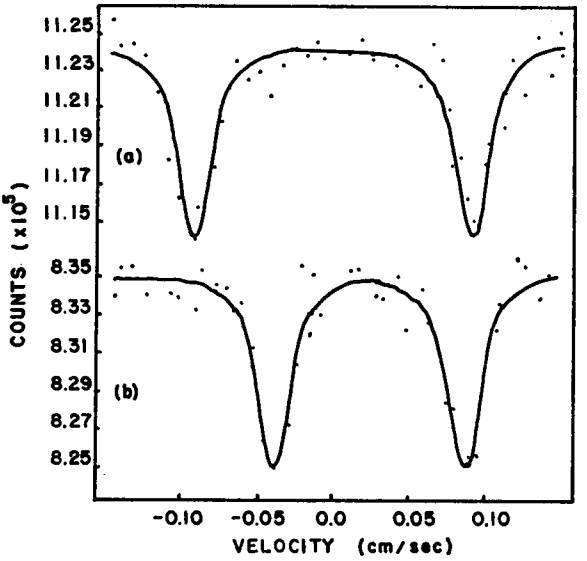

FIGURE 1 Mössbauer spectra of (a) ferricytochrome *c* and (b) ferrocytochrome *c* at 298°K.

In reporting their spectrum of cytochrome *c*, Maling and Weissbluth (2) noted that the negative peak was broader, less symmetric, and of a smaller amplitude than the positive velocity peak. They found, by optical absorption measurements, that the cytochrome *c* was a mixture of both the oxidized and reduced forms. Their suggestion that the broadening of the negative peak could be due to the mixture of the oxidized and reduced forms in their sample can be explained by the results shown in Fig. 1. Both the oxidized and reduced iron in the sample could contribute to the same positive peak. On the negative side of the spectrum, however, the oxidized and reduced portions of the sample would contribute to two peaks, separated by an interval of 0.50 ± 0.010 cm/sec. Therefore, it would be reasonable that these unresolved peaks would appear as a broader and less symmetric peak of smaller amplitude than the positive one. The data in Fig. 1, therefore, verify the conclusion reached

by Maling and Weissbluth, that the mixture of the two oxidation forms was the cause of the negative velocity line broadening in their spectrum.

The chemical shifts for ferri- and ferrocytochrome *c* are compatible with their assignments as low-spin complexes. The differential chemical shifts, δ_0, for ferric and ferrous inorganic complexes generally fall within the range of 0.00 to 0.04 cm/sec (9). Although our data indicate that the δ_0 of ferrocytochrome *c* is outside of this range, it is closer than to the range for high-spin ferrous compounds (0.140–0.155 cm/sec).

These data are in agreement with the results of the magnetic susceptibility studies of Theorell (10), who found that iron in both forms of cytochrome *c* is low-spin. Theorell examined aqueous solutions, and our results show that the iron in the cytochrome is also low-spin in the solid state. Gordy and Rexroad (11) suggest from the electron spin resonance of the oxidized form that the bonding of the iron is covalent.

The increase of δ_0 upon the reduction of the ferricytochrome *c* can be understood

TABLE I

QUADRUPOLE SPLITTINGS AND CHEMICAL SHIFTS OF FERRI- AND FERROCYTOCHROME *C* AT 298°K

Absorber	Quadrupole splitting	Chemical shift*	Differential chemical shift‡
	ΔE_Q	δ	δ_0
	cm/sec	cm/sec	cm/sec
Ferricytochrome *c*	0.190 ± 0.010	0.001 ± 0.005	0.044 ± 0.005
Ferrocytochrome *c*	0.129 ± 0.010	0.025 ± 0.005	0.069 ± 0.005

* Relative to ^{57}Co-Pd source.

‡ Relative to sodium nitroprusside.

in terms of the addition of an electron to the *d* orbital in the reduction of the iron from Fe^{III} to Fe^{II}. The chemical shift is related to the *s*-electron density at the iron nucleus. An estimate of the % 4*s*-electron contribution to the bonding can be obtained from the modified WWJ plot (9), which relates δ_0 to the 4*s* contribution to the iron bonding and the *s*-electron densities at the nucleus. The value obtained for the ferricytochrome *c* is approximately 10% and is increased upon reduction to approximately 35%. This indicates that the additional electron in ferrocytochrome *c* is in effect distributed in the molecular orbitals such as to increase the *s*-electron density at the iron nucleus. This suggests that the bonding of the ligands (Fig. 2) is different in the reduced state than in the oxidized state.

The electric quadrupole splitting found in ferricytochrome *c* (ΔE_Q = 0.190 cm/sec) corresponds to values found with low-spin Fe^{III} inorganic complexes, for example, $Na_2(Fe(CN)_5(H_2O)]$, 0.182; $Na_2[Fe(CN)_5NH_3]$, 0.178; and $Na_3 \cdot [Fe(CN)_5NO_2]$, 0.178 cm/sec (12). It is also compatible with the ΔE_Q of other low-spin Fe^{III} hemoproteins such as Hi-H_2O (0.200 cm/sec) and HiN_3(0.230 cm/sec)

reported by Lang and Marshall (8). The value of ΔE_Q for ferrocytochrome *c* (0.129 cm/sec) is larger than the values reported for low-spin Fe^{II} inorganic complexes. These values range between 0.070 and 0.089 cm/sec for pentacyanides where the other ligand is H_2O, NO_2^-, NH_3, and $SO_3^=$ (12). Although a large splitting is unusual for the inorganic complexes, there are significant exceptions to this tendency in biological samples. For example, oxyhemoglobin in which the iron is low-spin, has a large ΔE_Q (1).

In order to understand the unique Mössbauer spectra of hemoproteins, Bearden et al., examined a series of model heme compounds (5). Two of these ferrous compounds—bis(pyridine)-2,4-diacetyldeuteroheme dimethyl ester and bis(pyridine) mesoheme dimethyl ester—were identical except for the presence of ethyl groups at the 2 and 4 positions of the mesoporphyrin and acetyl groups in the 2 and 4 positions of the porphyrin. The porphyrin containing the acetyl groups was found to have a

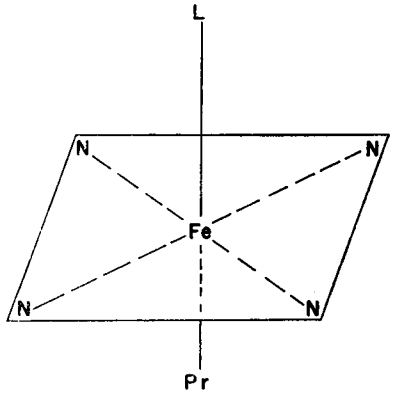

FIGURE 2 Structure of cytochrome *c* (diagrammatic), indicating Fe bonding to the porphyrin nitrogens (N) and the protein (L and Pr).

larger quadrupole splitting, 0.111 cm/sec at 77°K, than the porphyrin containing the ethyl groups. This latter compound had a ΔE_Q of 0.063 cm/sec. The δ_0 of these compounds were 0.053 and 0.050 cm/sec, respectively.

The quadrupole splitting of these two substances was attributed to the asymmetry of the ligand field (porphyrin ring). Furthermore, the magnitude of the splitting is dependent upon the degree of asymmetry. Since the acetyl groups show a larger electron-withdrawing effect than the ethyl groups, the difference between the porphyrin nitrogen bonds and the pyridine nitrogen bonds would be greater for the acetyl than the ethyl derivative. They concluded that the more electron-withdrawing are the groups on the porphyrin ring, the greater will be the nonequivalence between the porphyrin nitrogens (in-plane) and pyridine nitrogens (out-of-plane).

The chemistry and structure of cytochrome *c* has recently been reviewed by Margoliash and Schejter (13). The electronic spectra of both oxidized and reduced forms show that the iron is coordinated to nitrogenous groups. Recent results are in disagreement with the view that two histidine residues are coordinated to the iron.

The "crevice equations" formulated by George et al. (14) indicate that there is at least one histidine involved in ferricytochrome *c*. The heme iron shows a greater affinity for amino than for an imidazole nitrogen, and Margoliash and Frohwirt (15) suggested that the ligand, L (Fig. 2) might be an ϵ amino group from a lysine residue. Harbury and Loach suggested that a change from diimidazole to a mixed form might occur when cytochrome *c* is reduced from the oxidized state (16). Even if there are two imidazoles to the iron in both states, it is unlikely that they are of equal strength. In the crevice equations, there is an indication that one of the iron bonds to the protein is weaker than the other since the crevice can be opened by ligands with a 1:1 stoichiometry in a reversible fashion (17).

The Mössbauer data of cytochrome *c* are consistent with the view that the iron–protein bond is different than the iron–ligand bond (Fig. 2), since it suggests that the iron is in an asymmetric ligand field. The iron atom is in a more asymmetric field in the reduced state than in the oxidized state since ΔE_Q for ferrocytochrome *c* is greater than the values usually observed for low-spin ferrous complexes. The ΔE_Q for the ferricytochrome *c* corresponds to the values found with low-spin ferric complexes. The increase in the 4*s* electron density in the ferrocytochrome *c* implies a tighter geometry between the ligands and the iron atom giving rise to greater asymmetry than in ferricytochrome. The fact that there is no change in the chemical shift when the porphyrin ring of heme is altered (5), suggests that the major changes in the bonding upon reduction involves the ligands, L and Pr. This is not inconsistent with Orgel's (18) suggestion that the splitting of the degeneracy in the excited state is due to the asymmetry of the protein ligands (L and Pr) as well as the suggestion (15) of two different amino acid residues coordinated to the iron atom.

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