Mössbauer Spectroscopy of Bacterial Cytochromes*

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ABSTRACT: Mössbauer spectroscopy has been used to study the cytochromes *cc'* (RHP) and *c*-type cytochromes from the photosynthetic bacteria *Rhodospirillum rubrum* and *Chromatium*. The data for the proteins enriched in ^{57}Fe show that the two heme groups in the cytochromes *cc'* must be similar in iron coordination. The oxidized cytochromes *cc'* have a highly distorted high-spin electronic configuration, like that observed for methemoglobin. However, the signs of the electric field gradients are apparently opposite for methemoglobin and for the cytochromes *cc'*, indicating that the iron coordination is very different for the two proteins. The "thermally mixed" spin states attributed to these proteins are not observable as independent entities over the time (10^{-8} sec) characteristic of a Mössbauer measurement, but may be manifest in the temperature dependence of the quadrupole splitting. The cytochrome c_{552} from *Chromatium* has Mössbauer spectral characteristics very similar to those of the *c*-type cytochrome from *R. rubrum*, despite dissimilarities in redox potential, size, and CO binding ability. The two heme groups of the cytochrome c_{552} also appear identical in iron coordination. The cytochromes *cc'* are high-spin Fe^{2+} molecules in the reduced state, in contrast to the diamagnetic state of the *R. rubrum* cytochrome c_2 or the *Chromatium* cytochrome c_{552}. This fundamental difference in iron electronic configuration is another basis for considering the cytochromes *cc'* as a class distinct from *c*-type cytochromes.

The widely varying properties of heme proteins provide abundant evidence of the versatility of the iron–porphyrin complex as a biochemical unit. Mössbauer spectroscopy of proteins enriched in ^{57}Fe provides a means to study the immediate environment of the protein-bound iron. The configuration of the iron charge cloud and the effects of nearby ligands, transmitted either electrostatically or through delocalized molecular orbitals, are reflected in the Mössbauer spectrum (Wertheim, 1964; Fluck *et al.*, 1963; Bearden *et al.*, 1965b). Lyophilized mammalian cytochrome *c* has been used as a Mössbauer absorber (Maling and Weissbluth, 1964; Karger, 1964), but samples unenriched in ^{57}Fe had signal-to-noise ratios too low to allow effective interpretation.

The photosynthetic bacteria which can be readily enriched in ^{57}Fe offer several classes of heme proteins for study with the Mössbauer technique. The cytochrome *cc'* class[1] (Kamen, 1963), of which two members are examined in this study, is most distinctive in that it shows an optical absorption spectrum like that of hemoglobin and myoglobin, with a peak near 630 mμ in the oxidized form and broad absorption in the α region in the reduced form. However, the heme moiety in cytochrome *cc'* is covalently bound as in typical *c* cytochromes. Further, cytochrome *cc'* does not react with typical anionic ligands, such as azide, fluoride, or cyanide, although the heme is accessible to NO and to CO in the reduced state (Taniguchi and Kamen, 1963). The redox properties of cytochrome *cc'* are different from typical *c* cytochromes in that cytochrome *cc'*s have oxidation–reduction potentials around 0 V (Bartsch, 1963). The *cc'* proteins have magnetic susceptibilities near those of high-spin Fe(III) at room temperature (Ehrenberg and Kamen, 1965) and are also high spin at liquid nitrogen temperature (Tasaki *et al.*, 1967; Ehrenberg and Kamen, 1965).

Cytochrome c_{552} isolated from *Chromatium* strain D displays typical *c* cytochrome α and β bands, but with the α band at 552 mμ instead of the usual 550 mμ. This cytochrome is atypical in size with two hemes and one flavin, possibly FMN,[2] in a molecule of approximately 72,000 mol wt (Bartsch *et al.*, 1967). The oxidation–reduction potential of cytochrome c_{552} is +10 mV at pH 7.0, and this protein is reactive with CO (Bartsch, 1963). Cytochrome c_2 isolated from *Rhodospirillum rubrum* is a typical *c* cytochrome with respect to molecular weight (13,470), optical spectrum, oxidation–reduction potential ($E_{M,7}$ = 290–330 mV), and lack of reactivity with CO (Dus and Sletten, 1967; Bartsch, 1963).

Experimental Section

Cytochromes enriched in ^{57}Fe were isolated from cells grown in culture media in which the sole added

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[1] Formerly called *Rhodospirillum rubrum* heme protein (RHP).

[2] See *Biochemistry 5*, 1445 (1966), for list of abbreviations.

iron source was ^{57}Fe in the form of the chloride salt, prepared from $^{57}Fe_2O_3$ (92.5 atom % excess ^{57}Fe) obtained from Oak Ridge National Laboratory. For both *Chromatium* and *R. rubrum*, the media used were modifications of the heterotrophic medium ascribed to R. C. Fuller for anaerobic photosynthetic growth of *Chromatium* as described by Bose (1963). Succinic acid (4 g/l., Mallinckrodt, A. R.) was used as the organic substrate. As a source of trace elements, the trace element solution of Larsen (1952), from which all added iron salts were omitted, was used. For the *R. rubrum* medium, sodium sulfide was omitted, and 1 g/l. of Difco Bacto-Casamino Acids plus 10 μg/l. of biotin was added to the published mixture.

The endogenous level of iron in the two media was found to be 0.5 mg/l. To the *Chromatium* culture 2.9 mg/l. of ^{57}Fe was added and to the *R. rubrum* culture 3 mg/l. of ^{57}Fe was added. Therefore the final iron content of the media was approximately 80 atom % excess ^{57}Fe.

The *Chromatium* cytochromes were isolated from 373-g (wet weight) cells grown in 51 l. of the *Chromatium* medium under incandescent illumination (50–100 ft-candles) at 35°. The cells were suspended in 100 mM phosphate buffer (pH 7.5) (20% packed cells by weight) and were disrupted in a Servall-Ribi cell fractionator (Ivan Sorvall, Inc.). The extracted cell suspension was freed of cellular debris and cell membrane fragments by centrifugation for 1 hr at $3 \times 10^4 g$ and then 3 hr at $10^5 g$. To remove ferredoxin, the extract was passed through a DEAE-cellulose column (type 20, Brown Co.), equilibrated with the extraction buffer. The unadsorbed effluent from this column was desalted by passage through a G-25 Sephadex column and was then chromatographed on a DEAE-cellulose column equilibrated with 10 mM phosphate buffer (pH 7). The column was washed with a stepwise gradient consisting of this buffer plus an increasing concentration of sodium chloride. Cytochrome *cc'* was eluted by 12 mM NaCl, and cytochrome c_{552} was eluted by 16 mM NaCl. To concentrate the cytochromes, the separate fractions were diluted threefold with water and adsorbed on small columns of DEAE-cellulose. The main cytochrome *cc'* fraction, eluted with 90 mM NaCl, and the main cytochrome c_{552} fraction, eluted with 130 mM NaCl, were desalted, concentrated, and further purified by fractional precipitation with ammonium sulfate. The best cytochrome *cc'* fraction precipitated within the range 60–80% ammonium sulfate saturation at 5° with purity index $A_{280\,m\mu}/A_{400\,m\mu}$ 0.315. The best fraction of cytochrome c_{552} precipitated over the range of 50–80% ammonium sulfate saturation with purity index $A_{278\,m\mu}/A_{410\,m\mu}$ 0.530. Based on chromatographic behavior and spectroscopic properties (Bartsch, 1963) the two cytochromes were each uncontaminated by the other and were nearly homogeneous. Cytochrome c_{552} had evidently been partly depleted of flavin as compared with some more rigorously analyzed samples which had a purity index of 0.547 (Bartsch, 1963).

The ammonium sulfate precipitates were dialyzed against 5 mM sodium phosphate buffer (pH 7) plus 0.1 mM disodium EDTA; then the samples were concentrated by inverse dialysis against dry G-200 Sephadex. Aliquots of 0.3 ml each of cytochrome *cc'*, containing 3.7 μmoles/ml, and cytochrome c_{552}, containing 2.9 μmoles/ml, were transferred to Mössbauer spectrometer cuvets.

R. rubrum cytochromes were isolated from 278-g (wet weight) cells grown in 68 l. of the *R. rubrum* medium. A cell-free extract was prepared and the ferredoxin was removed in the manner already described, but with 100 mM Tris buffer (pH 7.8) used as the extracting solvent. The cytochrome solution so obtained was desalted with the aid of G-25 Sephadex and then was chromatographed on a DEAE-cellulose column equilibrated with 1 mM Tris buffer (pH 8) (unpublished method of R. G. Bartsch and T. Horio). Cytochrome c_2 was eluted with 30 mM Tris buffer and cytochrome *cc'* was eluted with 50 mM Tris buffer. Rechromatography on DEAE-cellulose, followed by fractional precipitation with ammonium, yielded the following cytochrome samples: 1.7 μmoles of cytochrome c_2 ($A_{272\,m\mu}/A_{415\,m\mu}$ 0.23) and 0.68 μmole of cytochrome *cc'* ($A_{282\,m\mu}/A_{390\,m\mu}$ 0.42). The cytochrome c_2 and cytochrome *cc'* were completely separated. Based on spectroscopic properties (Bartsch, 1963) cytochrome c_2 was nearly homogeneous. However, cytochrome *cc'* was not free of colorless proteins, inasmuch as homogeneous preparations ordinarily attain a purity index equal to 0.28. Further purification of the sample was not attempted in order to conserve the limited amount of ^{57}Fe-enriched cytochrome.

The proteins were precipitated with nearly saturated ammonium sulfate directly into the Mössbauer spectrometer cuvet holders, from which the supernatant fluid was removed after centrifuging the cytochrome to the bottom of the holders. Finally, the samples were dissolved in 0.1–0.3 ml of water. Mössbauer spectra of precipitated and frozen solutions of the dissolved proteins were nearly identical. In all the experiments between 1 and 2 μatoms of ^{57}Fe were present in the sample.

All measurements were made on frozen solutions or frozen precipitates of the protein. This is worth noting in view of evidence that in some cases protein magnetic properties, for instance, can change reversibly on freezing (Ehrenberg and Estabrook, 1966). However, in the cases of the cytochromes *cc'* and the cytochrome c_{552}, independent magnetic susceptibility or electron resonance experiments have shown that the magnetic properties are the same in the frozen state as in liquid solution (Ehrenberg and Kamen, 1965; Tasaki *et al.*, 1967). For the *R. rubrum* cytochrome c_2 no such magnetic measurements exist, but the Mössbauer results at low temperature are fully compatible with the low-spin configuration typical of *c*-type cytochromes in liquid solution. These independent checks on the similarity of the iron coordination in the frozen state and in liquid solution are the basis of the assumption that Mössbauer data can be compared with other data in the literature.

The *Chromatium* cytochromes and *R. rubrum* cyto-

TABLE I: Mössbauer Parameters for *Chromatium* and *R. rubrum* Cytochrome *cc'*.

		Oxidized		Reduced		CO	
	°K	*R. rubrum*	*Chromatium*	*R. rubrum*	*Chromatium*	*R. rubrum*	*Chromatium*
Quadrupole splitting, ΔE, ± 0.02 mm/sec	205	1.56	1.97	1.44	1.34	0.36	0.28
	77	1.78	2.27	1.88	1.77	0.36	0.29
	63	1.94	2.53		1.91		
	4.6	Diffuse hyperfine splitting		2.05	1.97	0.36	0.28
Isomer shift, δE (relative to Fe:Cu), ± 0.02 mm/sec	205	0.06	0.08	0.52	0.52	−0.05	−0.06
	77	0.08	0.11	0.58	0.59	0.00	0.02
	63	0.08	0.14		0.59		
	4.6	Diffuse hyperfine splitting		0.59	0.57	−0.01	−0.02

TABLE II: Mössbauer Parameters for *Chromatium* Cytochrome c_{552} and *R. rubrum* Cytochrome c_2.

		Oxidized		Reduced		CO
	°K	*R. rubrum*	*Chromatium*	*R. rubrum*	*Chromatium*	*Chromatium*
ΔE (±0.01 mm/sec)	205	2.16	2.22	1.14	1.32	0.37
	77	2.27	2.14	1.13	1.26	0.38
	4.6	Diffuse hyperfine splitting		1.12		
δE (±0.02 mm/sec)	205	−0.08	−0.05	0.14	0.13	−0.09
	77	−0.05	−0.03	0.18	0.19	−0.05
	4.6	Diffuse hyperfine splitting		0.19		

chrome *cc'* were obtained in the completely oxidized state. Cytochrome c_2 was obtained in the reduced state. After the latter cytochrome had been examined in the reduced form, the sample was removed from the cuvet, desalted, and passed through a 1 × 1 cm column of Amberlite IR 400 anion-exchange resin charged with sodium ferricyanide to oxidize the heme protein without addition of contaminant iron compounds. The sample was then reprecipitated with ammonium sulfate in the cuvet and examined in the oxidized state. The other cytochromes were reduced by the addition of 0.5–1 mg of sodium dithionite directly to the solutions in the cuvet holders. After the Mössbauer spectra of the reduced samples had been measured, these solutions were equilibrated for several hours with 1 atm of carbon monoxide. The cuvets were mounted in Thunberg tubes which were alternately evacuated and then filled with CO to remove air. The prolonged equilibration time was required to permit completion of the slow diffusion of the CO into the viscous protein solutions. The extent of the reductions or formation of carbon monoxide complexes was monitored by measuring the optical spectra of 10–15-μl portions of the concentrated solutions sucked directly into an optical cuvet with 30-μm optical path length (obtained from Limit Corp., Division of Beckman Instruments, Inc.). The Mössbauer spectrometer used in these experiments has been previously described (Bearden *et al.*, 1965a).

Results

Mössbauer spectra were obtained over a temperature range of 4.6–270°K; results are shown in Tables I and II. The quadrupole splitting, ΔE, increases with the deviation of the iron electron cloud from spherical symmetry, as caused by the configuration of the d shell, or by the ligand field. The isomer shift, δE, decreases with increasing s-electron density at the nucleus, as determined by d-shell shielding, or by the s character of molecular orbitals involving iron electrons (Walker *et al.*, 1961). ΔE is measured from the observed spectrum as the splitting of the absorption lines. δE is the energy difference between the position of the center

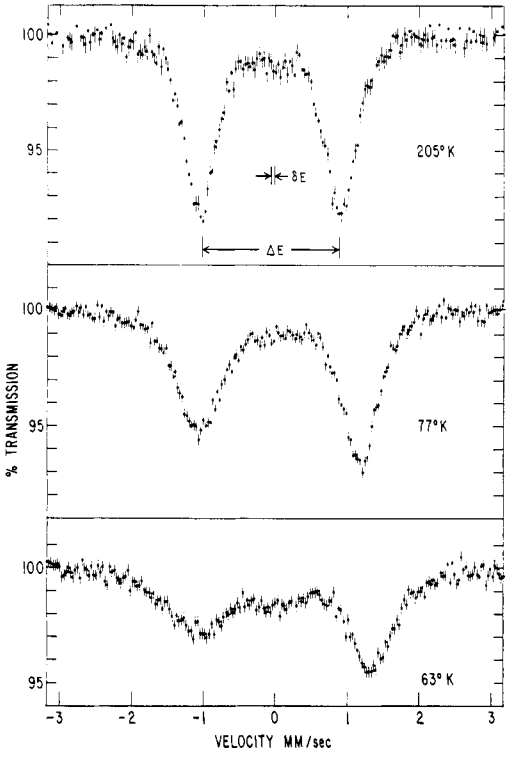

FIGURE 1: Mössbauer absorption spectra of oxidized *Chromatium* cytochrome *cc'* at 205, 77, and 63°K.

of symmetry of the absorption spectrum and zero absorber velocity relative to a source of ^{57}Co in copper metal. The temperature dependence of the line widths indicates the extent of interactions of the nuclear spin with unpaired electrons (Bradford and Marshall, 1966).

The Mössbauer spectrum of oxidized *Chromatium* cytochrome *cc'* at three temperatures is shown in Figure 1. The lines broaden as the temperature is lowered, with the lower energy line broadening first.

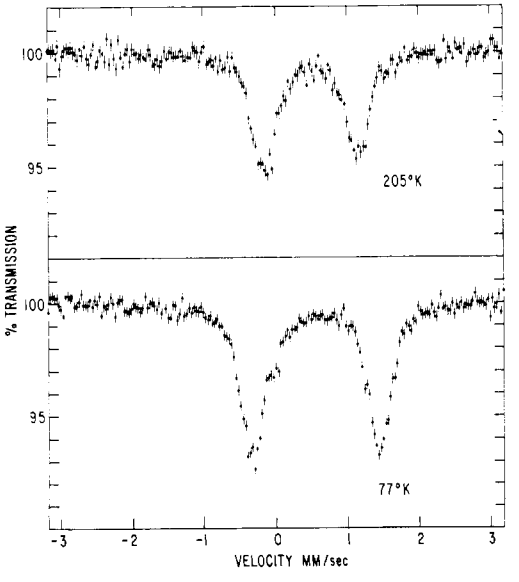

FIGURE 2: Mössbauer absorption spectrum of reduced *Chromatium* cytochrome *cc'* at 205 and 77°K.

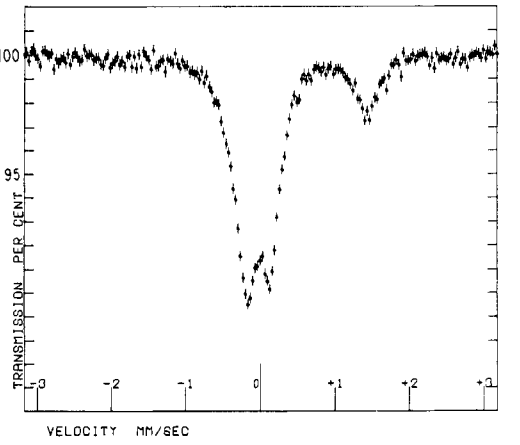

FIGURE 3: Mössbauer absorption spectrum of *Chromatium* cytochrome *cc'*–CO complex at 77°K. The absorption at +1.5 mm/sec coupled with its opposite member at −0.1 mm/sec represents unreacted reduced cytochrome *cc'* (compare with Figure 2).

At 4.6°K the quadrupole doublet breaks completely into diffuse hyperfine broadening. The line broadening as the temperature is lowered indicates that the relaxation time of the effective magnetic field at the ^{57}Fe nucleus due to unpaired iron electrons is becoming comparable to the characteristic time (10^{-8} to 10^{-7} sec) of the Mössbauer hyperfine interaction (Bradford and Marshall, 1966).

Both *Chromatium* and *R. rubrum* cytochrome *cc'* (the Mössbauer parameters are given in Table I) show the same general Mössbauer spectra and response to temperature; however, *R. rubrum* cytochrome *cc'* shows a narrower quadrupole splitting than does the *Chromatium* protein. Both of the cytochromes *cc'* resemble methemoglobin (Lang and Marshall, 1966a) in having quadrupole splitting in the oxidized state which is much greater than that of model Fe^{3+} compounds (Fluck *et al.*, 1963).

Figure 2 gives the spectrum of reduced *Chromatium* cytochrome *cc'*, which is typical of high-spin Fe^{2+} compounds (Fluck *et al.*, 1963), in agreement with magnetic susceptibility measurements (Ehrenberg and Kamen, 1965). The increased isomer shift of the reduced spectrum relative to the oxidized spectrum results from less s-electron density at the iron nucleus, due to shielding by the extra d electron (Wertheim, 1964).

Figure 3 shows the spectrum of Fe^{2+} *Chromatium* cytochrome *cc'* with CO bound as a ligand. The small amount of absorption at +1.5 mm/sec, coupled with its opposite member at −0.1 mm/sec, represents unreacted reduced cytochrome *cc'*. The 10% of the total intensity in this wide pair agrees with the optical spectrum of this sample, which indicates only 90% conversion into the CO form. The CO form of *R. rubrum* cytochrome *cc'* is quite similar to that observed for *Chromatium* cytochrome *cc'* (Table I).

Figures 4–6 show the Mössbauer spectra of the oxidized, reduced, and reduced-CO forms of *Chromatium* cytochrome c_{552}; the Mössbauer parameters for this protein are given in Table II. In the oxidized forms

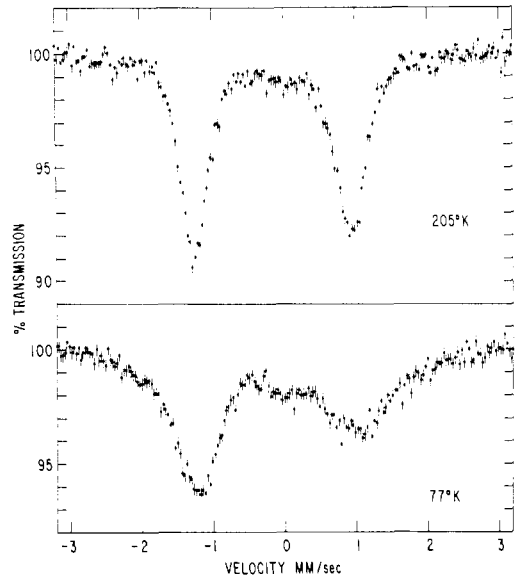

FIGURE 4: Mössbauer absorption spectrum of oxidized *Chromatium* cytochrome c_{552} at 205 and 77°K.

both cytochrome c_{552} and the *R. rubrum* cytochrome c_2 show a widely split quadrupole doublet. The splitting remains essentially constant with decreasing temperature. As with cytochrome *cc'*, both *c*-type cytochromes show a broadening of absorption lines with decreasing temperature, resulting in diffuse absorption at 4.6°K. The small isomer shift (0.2 mm/sec) and quadrupole splittings of the reduced *c*-type cytochromes are compatible with the expected low-spin Fe^{2+} heme coordination.

The Mössbauer spectrum of the CO form of cytochrome c_{552} is similar to those of the cytochrome *cc'*–CO derivatives. The absorption at 0.8 mm/sec coupled with a portion of the absorption at −0.5 mm/sec probably results from unreacted Fe^{2+} cytochrome c_{552}. Too little sample was available to judge from the optical spectrum of the concentrated material, whether the reaction was complete, but it was found in the case of the cytochrome *cc'* that reactions with CO might not go to completion in as much as 12 hr with the high protein concentration used.

Discussion

Cytochrome cc' Proteins. The split Soret band of both cytochromes *cc'* (Bartsch, 1963) and the fact that the *Chromatium cc'* has two hemes but only three histidines (Bartsch *et al.*, 1961) have raised the possibility that the iron-bound ligands may not be the same for the two hemes in *R. rubrum* and *Chromatium* cytochromes *cc'*. However, the Mössbauer spectra of all forms of both *R. rubrum* and *Chromatium* cytochrome *cc'* display only a single pair of Mössbauer absorption lines,[3] instead of two pairs which might be expected if two distinct iron coordinations existed in the protein.

[3] In the oxidized form, the lines are broadened by magnetic effects at lower temperatures.

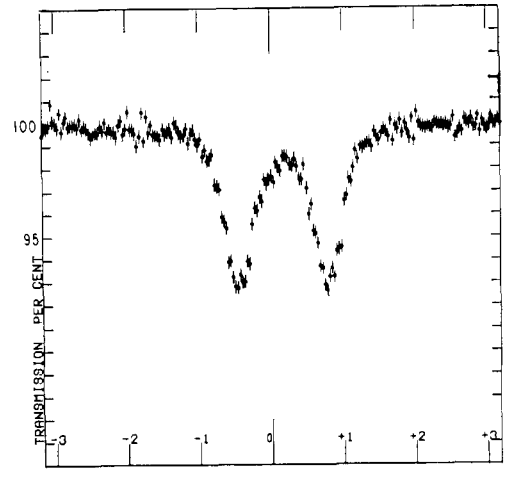

FIGURE 5: Mössbauer absorption spectrum of reduced *Chromatium* cytochrome c_{552} at 77°K.

Mössbauer studies with high-spin model heme compounds have shown that quadrupole splittings vary as much as 0.7 mm/sec over the series of ions (Cl^-, Br^-, I^-, F^-, N_3^-, and CH_3COO^-) as perpendicular ligands (T. H. Moss, A. J. Bearden, and W. S. Caughey, to be published). These studies give an indication of the sensitivity of the heme quadrupole splitting to the electronegativity or π-bonding ability of the iron-bound ligand. Within this sensitivity, the failure to observe two distinct pairs of quadrupole lines suggests that the iron coordination of the two hemes in the cytochromes *cc'* is closely similar.

It is worth noting the substantial (20%) difference in quadrupole splitting between the *R. rubrum* and *Chromatium* cytochromes *cc'*. Because of their close chemical similarities (Bartsch, 1963) it is unlikely that different ligands are coordinated to the iron in the two proteins. This suggests, then, that the Mössbauer spectra of these cytochromes *cc'* may be able to resolve differences in iron environment more subtle than the

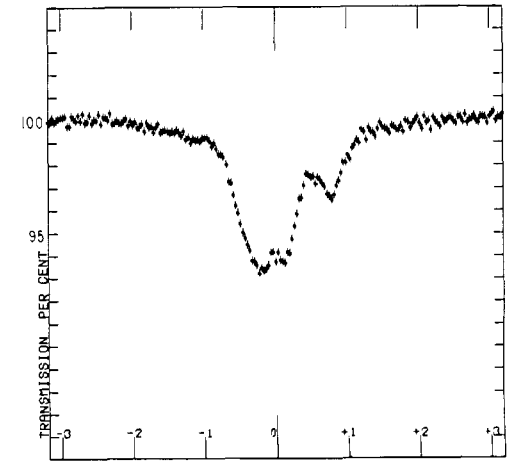

FIGURE 6: Mössbauer absorption spectrum of *Chromatium* cytochrome c_{552}–CO complex at 77°K. The absorption at +0.8 mm/sec coupled with its opposite member at −0.5 mm/sec results from unreacted reduced cytochrome c_{552} (compare with Figure 5).

immediate coordination sphere. Lack of sufficient model studies prevents us from making any specific suggestions as to these differences.

Owing to the similarities in optical spectra and magnetic properties, it is of interest to compare the Mössbauer spectra of the cytochromes *cc'* with that of methemoglobin (Lang and Marshall, 1966a,b). These proteins all exhibit quadrupole splittings (>2.0 mm/sec) in the oxidized state which are much wider than those (<1.0 mm/sec) observed for typical high-spin Fe^{3+} compounds (Fluck *et al.*, 1963). This has led one of the authors to question whether methemoglobin, under certain experimental conditions, could actually be a low-spin form (Moss, 1966). Similarly, it has been speculated (Bearden *et al.*, 1966) that the cytochromes *cc'* might be a case of the unusual $S = {}^3/_2$ spin state. However, subsequent experiments have shown that when the model compound hemin chloride ($\Delta E \approx 0.8$ mm/sec) is dissolved in pyridine, a compound is formed which has a magnetic susceptibility close to high spin, a $g_\perp = 6$ electron spin resonance signal, and a wide quadrupole splitting ($\Delta E \approx 1.8$ mm/sec) near that of methemoglobin and cytochrome *cc'* (T. H. Moss, A. J. Bearden, and W. S. Caughey, to be published). The wide quadrupole splitting may be due to formation of a six-coordinated compound, in which the iron is more nearly in the porphyrin plane, and interacts more strongly with the porphyrin nitrogens, than is the case in the pentocoordinated compounds. In any case it confirms the view (Williams, 1966; Lang and Marshall, 1966b) that wide quadrupole splitting cannot be considered anomalous for high-spin Fe^{3+} coordination.[4] Methemoglobin and the cytochromes *cc'* thus belong to a class of high-spin Fe^{3+} coordinations in which the nominally symmetric iron electronic configuration is highly distorted by interactions with neighboring ligands.

Though the proteins have in common a wide quadrupole splitting, there is one important difference between the wide split pair of lines in the cytochrome *cc'* spectra and those in the methemoglobin spectrum. The higher energy quadrupole line (higher energy is toward positive source velocity) in the hemoglobin spectrum broadens most rapidly as the temperature is lowered,[5] while for both cytochromes *cc'* the lower energy line broadens most rapidly. Interaction between unpaired electron spins and the nuclear spin is the cause of the broadening; whether the higher or lower energy line is most broadened in cylindrical symmetry depends on the sign of the nuclear quadrupole coupling constant (Blume, 1965; Bradford and Marshall, 1966). The assumption of cylindrical symmetry here is reasonable because both cytochromes *cc'* show a $g = 6$ electron spin resonance signal typical of axially symmetric Fe^{3+} heme proteins (Ehrenberg and Kamen, 1965). The fact that opposite lines are broadened in the methemoglobin and cytochrome *cc'* spectra suggest that the symmetry of the iron charge clouds, and the iron coordination, are different. The apparent negative nuclear quadrupole coupling constant (or field gradient; see Grant (1966) for comparison of this notation) for both cytochromes *cc'* is compatible with a configuration where the concentration of charge along the z axis is increased relative to that in the x–y plane, by occupancy of ligand electrons in molecular orbitals of considerable metal d_π or d_{z^2} character.

Methemoglobin hydroxide and the cytochromes *cc'* have been considered as examples of heme proteins which exist in "thermal mixtures" of electronic spin states (Ehrenberg and Kamen, 1965; George *et al.*, 1964). Yet none of these display more than one pair of Mössbauer quadrupole lines over the temperature range 4–270°K. These data put constraints on the thermal mixture interpretation by showing that the individual $S = {}^1/_2$ and ${}^5/_2$ electronic states do not exist for times as long as $\approx 10^{-8}$ sec, the time required for a Mössbauer measurement (Lang and Marshall, 1966b; Williams, 1966). However, for both methemoglobin (Lang and Marshall, 1966b) and the cytochromes *cc'*, the magnitude of the quadrupole splitting is much more temperature dependent than is usual for pure high-spin Fe^{3+} compounds (Fluck *et al.*, 1963). This observation is compatible with the existence of a thermal mixture of short-lived ($<10^{-8}$ sec) electronic states. The quadrupole splitting for this mixture of short-lived states would be determined by the weighted average of the field gradients due to the individual components, and would change with temperature. Though more examples should be studied, the comparison of the hemoglobin and cytochrome *cc'* data suggests that phenomena associated with "mixed-spin states" in Fe^{3+} heme proteins, such as temperature-dependent optical absorption near 630 mμ and intermediate magnetic susceptibilities, will be manifest only as temperature-dependent quadrupole splittings in Mössbauer spectroscopy.

The temperature dependence of the quadrupole splitting of the reduced cytochromes *cc'* also indicates a thermal mixture of electronic states, but in this case the components do not differ in magnetic properties. This variation with temperature has been observed for many model high-spin Fe^{2+} compounds (Ingalls, 1964) and is due to the fact that the sixth d electron will occupy the closely spaced three lowest d orbitals in a Boltzman temperature distribution. The temperature dependence of the quadrupole splitting will then be described by Boltzman functions. By picking the parameter Δ for the splitting of the d orbitals which enables the Boltzman function to best match the experimental points, we can make an estimate of this splitting in the protein (Ingalls, 1964). Chemically,

[4] It is clear from this and many other observations that, though they often show characteristic Mössbauer spectra, high- and low-spin Fe^{3+}, and low-spin Fe^{2+}, can be similar enough so that Mössbauer data alone cannot distinguish among them. High-spin Fe^{2+} can usually be identified as a consequence of the large positive isomer shift and the generally large temperature-dependent quadrupole splitting.

[5] The sign of the right side of eq 18 in Lang and Marshall (1966a) is in error (G. Lang, personal communication), confusing the following discussion of the sign of the field gradient. The best way to see the difference between methemoglobin and the cytochromes *cc'* is to compare Figure 18 of Lang and Marshall with Figure 1 of this work.

this Δ is significant in that it is determined to a large extent by the π-bonding characteristics of the iron-bound ligand.

The negative nuclear quadrupole coupling constant indicates that in cylindrical symmetry the iron d_π (d_{yz} orbitals) lie lowest (Ingalls, 1964). Using the observed 4.6°K splitting as representing the quadrupole splitting ΔE_{d_π} due to a single electron occupying a d_π orbital, and using the fact that $\Delta E_{d_\pi} = -1/2\Delta E_{d_{xy}}$ (Ingalls, 1964), we can write the temperature dependence of the reduced protein quadrupole splitting as indicated in eq 1. Δ represents the splitting of the lower three

$$\Delta E(T) = \frac{2\Delta E_{d_\pi} + \Delta E_{d_{xy}} e^{-\Delta/kT}}{2 + e^{-\Delta/kT}} = \frac{2\Delta E_{4.6°K}(1 - e^{-\Delta/kT})}{2 + e^{-\Delta/kT}} \quad (1)$$

(d_ϵ) d orbitals shown in Figure 7. The best fit for both *Chromatium* and *R. rubrum* cytochrome *cc'* uses $\Delta = (250 \pm 20)$°K. The theoretical curve for the *Chromatium* data is plotted (solid line) together with the observed quadrupole splittings in Figure 7. Though we have not considered departures from cylindrical symmetry this Δ is a measure of the π-bonding interactions of the iron orbitals, and should be considered in judging speculations concerning the iron-bound ligands of cytochrome *cc'*. The value 250°K (362 cm^{-1}) is in fact much smaller than values obtained for the splitting of the d_π orbitals in strongly π-bonded compounds like $K_3Fe(CN)_6$ (Shulman and Sugano, 1965). Iron-bound ligands with little tendency to form d_π bonds with heme iron are thus most likely for the cytochromes *cc'*.

The CO complexes of the cytochromes *cc'* show a hemochromogen-type visible absorption spectra typical of low-spin heme proteins (George *et al.*, 1964). The Mössbauer spectrum, with small (<0.5 mm/sec), temperature-independent quadrupole splitting, and small (<0.3 mm/sec) isomer shift, is fully compatible with a low-spin Fe(II) form. Similar results have been obtained for model low-spin heme complexes (Bearden *et al.*, 1965b) and other low-spin Fe^{2+} compounds when the iron coordination is such as to permit formation of d_ϵ molecular orbitals of considerable ligand character (Shulman and Sugano, 1965).

c-Type Cytochromes. The similarity of the Mössbauer spectra of the oxidized and reduced *Chromatium* cytochrome c_{552} to the *R. rubrum* cytochrome c_2 emphasizes the relation of the c_{552} to *c*-type cytochromes, although the two have widely different properties with respect to size, ability to bind CO, and redox potential. In the oxidized form both show a wide (<2.0 mm/sec) quadrupole splitting, small isomer shift, and temperature-dependent line broadening as seen with other low-spin Fe(III) heme proteins (Lang and Marshall, 1966a). The lack of temperature dependence of the quadrupole splitting between 77 and 250°K indicates that the d_π and d_{xy} orbitals must be split by $\gtrsim$300°K (Ingalls, 1964). This large splitting is as expected for perpendicular ligands with strong covalent bonds to the iron. The broadening of the higher energy line indicates a positive

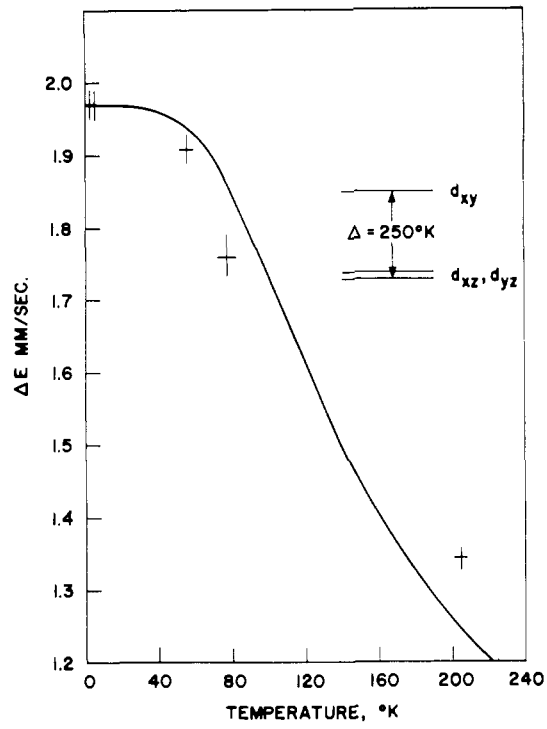

FIGURE 7: Temperature dependence at the quadrupole splitting of reduced *Chromatium* cytochrome *cc'*. The solid line is the theoretical curve for a low-spin configuration with the d_π and d_{xy} orbitals split by 250°K.

electric quadrupole coupling constant, and thus the d_{xy} orbital lies lowest. As with the cytochromes *cc'*, there is only one pair of absorption lines, so that there is no difference detectable in these Mössbauer spectra between the two hemes.

The small isomer shift (+0.2 mm/sec) and temperature-independent quadrupole splittings of the reduced cytochromes are again typical of the low-spin Fe(II) heme coordination (Bearden *et al.*, 1965b). This result is as expected from optical spectroscopic similarities with other *c*-type cytochromes (Bartsch, 1963). This is an important point of contrast with reduced cytochromes *cc'*, which are clearly high spin as judged by both these Mössbauer studies and magnetic measurements (Ehrenberg and Kamen, 1965).

The portion of the cytochrome c_{552} which has reacted with CO in our sample (the narrowly split absorption in Figure 6) has a spectrum once again compatible with the expected low-spin Fe^{2+} configuration. Lack of model studies indicating sensitivity of this configuration to changes in iron-bound ligand prevents us from making any definite conclusions about the strong similarity of the CO–cytochrome c_{552} spectrum and the CO–cytochrome *cc'* spectra.

The most striking difference seen in this study between the cytochromes *cc'* and the *c*-type proteins is the electronic state of the reduced forms. The distinct high-spin Fe^{2+} state of the cytochromes *cc'* indicates a weak ligand field environment for the iron (Orgel, 1960). The typical low-spin hemochronogen behavior of the two reduced *c*-type proteins suggests a contrasting

strong ligand field. This provides another criterion for distinguishing the *cc'* class of cytochromes.

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