Charge-Transfer Optical Spectra, Electron Paramagnetic Resonance, and Redox Potentials of Cytochromes†

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ABSTRACT: Near-infrared optical absorption spectra were measured for a series of ferric low-spin complexes of cytochrome *c* and myoglobin. The frequency of the first two charge-transfer bands, previously assigned to $a_{2u}(\pi)$,$a_{1u}(\pi) \rightarrow$ iron (d_{yz}) orbital promotions, was found to be linearly proportional to the iron d_{yz}–d_{xy} orbital energy difference, as calculated from published electron paramagnetic resonance spectra. This result indicates that the change in the charge-transfer frequency is mainly determined by the change in the d_{yz} orbital energy. The d_{yz} orbital accepts the electron in the chemical reduction of ferricytochrome *c*, and it is proposed that differences in charge-transfer frequency among cytochromes may be useful to estimate the contribution of the axial ligands, relative to the protein environment, toward determining differences in oxidation–reduction potentials.

Since the original applications of ligand field theory to heme proteins in the 1950s by P. George (George et al., 1956, 1961) and J. S. Griffith (Griffith, 1957), there have been numerous investigations of their optical [Dolphin (1978) and references cited therein; Eaton & Hofrichter, 1981] and electron paramagnetic resonance (EPR) spectra (Palmer, 1978; Blumberg, 1981). There has, however, been no theoretically based correlation of the results obtained by the two spectroscopic methods, and no theoretical connection has been made between spectroscopic and thermodynamic parameters. The most straightforward correlation would be between the ligand field parameters obtained from the optical frequencies of the appropriate d → d transitions and those calculated from the *g* values measured by EPR. Up to now, however, d → d transitions have only been observed in the ferrous state (Eaton & Charney, 1969; Eaton et al., 1978; Eaton & Hofrichter, 1981), which does not exhibit an EPR spectrum. Furthermore, all d → d transitions for high-spin ferric heme complexes are spin forbidden, while for low-spin ferric complexes the transitions between the relevant $d(t_2)$ orbitals are predicted to be in the vibrational infrared (Eaton & Hofrichter, 1981). Here we report a novel correlation between the frequencies of the lowest energy charge-transfer transitions, assigned as $a_{2u}(\pi)$,$a_{1u}(\pi)$ → iron (d_{yz}), and the orbital splitting parameters calculated from the *g* values for a series of ferric low-spin complexes of cytochrome *c*, myoglobin, and hemoglobin. The correlation is readily explained with the simplest theoretical model for the optical transitions, indicating that the change in charge-transfer frequency among the series is mainly determined by a change in the energy of the d_{yz} orbital. Since the d_{yz} orbital accepts the electron in the chemical reduction of ferricytochrome *c*, this finding suggests that the contribution to the change in oxidation–reduction potential of cytochromes resulting from a change in axial ligand can be estimated from the difference in charge-transfer frequency.

Materials and Methods

Horse heart cytochrome *c*, purchased from Sigma Chemical Co. (type VI), was purified by column chromatography on Amberlite CG-50. The dicarboxymethylated form, in which the methionines in positions 65 and 80 are carboxymethylated, was prepared as described previously (Schejter & Aviram, 1970). Sperm whale skeletal muscle myoglobin, purchased from Sigma Chemical Co. (type III), was purified by column chromatography on DEAE-cellulose. The proteins were incubated in 99.7% D_2O, lyophilized, and redissolved in D_2O containing 0.1 M potassium phosphate, pD 7.0. Complexes were formed by the addition of potassium cyanide, potassium azide, or imidazole. Absorption spectra were measured with a Cary 17 spectrophotometer interfaced to a Hewlett-Packard 9825A calculator.

Results and Discussion

Near-infrared optical absorption spectra were measured in D_2O at room temperature for native ferricytochrome *c* at neutral pD, for ferricytochrome *c* in 1 N NaOD, and for the cyanide, imidazole, and azide complexes of both ferricytochrome *c* and ferrimyoglobin at neutral pD. For the azide and imidazole complexes, the spectra of dicarboxymethylated cytochrome *c* were also measured, since complex formation proceeds to a much greater extent than with the unmodified protein (Schejter & Aviram, 1970). High-resolution nuclear magnetic resonance studies showed that for cytochrome *c* both complex formation and dicarboxymethylation displace the methionyl ligand (Wüthrich, 1969; Wüthrich et al., 1971), so that in all of these molecules one of the axial ligands is the imidazole of histidine.

Figure 1 shows the near-infrared optical absorption spectra of native cytochrome *c* and its complexes with imidazole and hydroxide. Table I summarizes the optical and EPR data for all the complexes. It contains the frequencies of the first two absorption maxima, the observed and calculated *g* values, and the values for the quantities Δ_1/λ and Δ_2/λ, where λ is the spin–orbit coupling constant and Δ_1 and Δ_2 are the iron d-orbital splitting parameters defined by the energy level diagram:

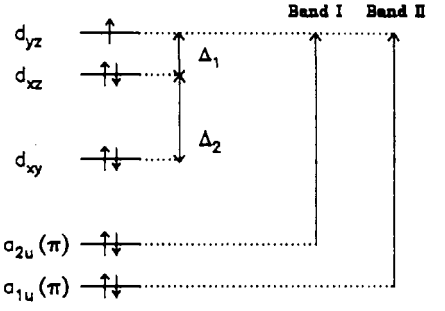

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Table I: Electron Paramagnetic Resonance and Near-Infrared Optical Spectra of Ferric Low-Spin Derivatives of Cytochrome *c*, Myoglobin, and Hemoglobin

compd[a]	EPR results[b]					optical maxima (cm^{-1})[c]	
	g_x	g_y	g_z	Δ_1/λ	Δ_2/λ	ν_I	ν_{II}
cyt *c*	1.25[d] (1.25)	2.25 (2.25)	3.06 (3.06)	1.48	1.82	5780	6825
cyanocyt *c*	0.93[e] (0.92)	1.89 (1.86)	3.45 (3.40)	0.93	2.87	6410[f]	7750
cyano-Mb	0.93[f] (0.92)	1.89 (1.86)	3.45 (3.40)	0.93	2.87	6410	7750
azidocyt *c*	1.81[g] (1.75)	2.30 (2.26)	2.73 (2.68)	2.77	2.77	7870[h]	9000
azido-Mb	1.72[f] (1.70)	2.22 (2.21)	2.80 (2.78)	2.38	3.44	7820	
azido-Hb	1.69[i] (1.70)	2.18 (2.19)	2.78 (2.79)	2.35	3.75	7820	8800
imidazolocyt *c*	1.58[g] (1.53)	2.30 (2.26)	2.96 (2.90)	1.95	2.37	6580[h]	7840
imidazolo-Mb	1.53[f] (1.53)	2.26 (2.26)	2.91 (2.91)	1.93	2.34	6670	7690
cyt *c* (1 N NaOD)	1.85[e] (1.85)	2.18 (2.18)	2.56 (2.56)	3.47	4.76	8430	9520

[a] All solutions at neutral pD, except cytochrome *c* in 1 N NaOD. [b] For each complex is given the observed *g* value, the *g* value calculated as described in the text (in parentheses), and the iron d-orbital splitting parameters, Δ_1/λ and Δ_2/λ, calculated as described in the text. [c] All optical data are taken from this work, except the azide complex of ferrihemoglobin that was taken from Stephens et al. (1976). [d] Salmeen & Palmer, 1968. [e] Brautigan et al., 1977. [f] Hori, 1971. [g] Ikeda-Saito & Iizuka, 1975. [h] Complex formed with dicarboxymethylferricytochrome *c*. [i] Yonetani et al., 1971.

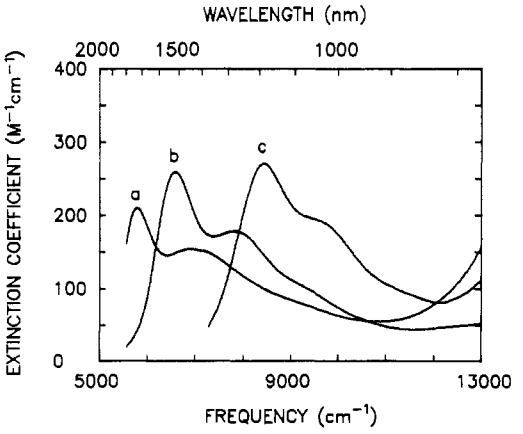

FIGURE 1: Near-infrared optical absorption spectra of ferricytochrome *c* complexes in D_2O at room temperature (2 mM, 1-cm path). Spectrum a is ferricytochrome *c* in 0.1 M potassium phosphate (pD 7.0), spectrum b is the cyanide complex in 0.1 M potassium phosphate (0.1 M KCN, pD 7.4), and spectrum c is ferricytochrome *c* in 1 N NaOD. The curves are plots of the measured optical densities at 1.0-nm intervals connected by straight lines. The contribution to the observed spectrum from HOD and protein vibrational absorption was subtracted by using the spectrum of ferrocytochrome *c*, which has very weak electronic absorption in the 1000–1800-nm wavelength range.

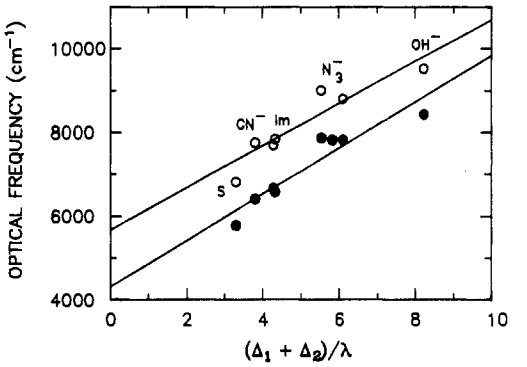

FIGURE 2: Near-infrared transition frequencies of ferricytochrome *c*, ferrimyoglobin, and ferrihemoglobin low-spin complexes vs. the orbital splitting parameter $(\Delta_1 + \Delta_2)/\lambda$, calculated from EPR measurements as described in the text. Data are from Table I. The straight lines are from a least-squares analysis and correspond to the first (filled circles, slope = 550 ± 70 cm^{-1}, intercept = 4320 ± 370 cm^{-1}) and second (open circles, slope = 500 ± 80 cm^{-1}, intercept = 5680 ± 410 cm^{-1}) near-infrared bands, ν_I and ν_{II}.

The values for Δ_1/λ and Δ_2/λ were calculated for a low-spin ferric ion in an octahedral crystal field with tetragonal and rhombic distortions from the relations (Kotani, 1961; Weissbluth, 1974):

$$\Delta_1/\lambda = (b + c)/(2a) + (c - a)/(2b)$$

$$\Delta_2/\lambda = (b - a)/(2c) - (c - a)/(2b) \quad (1)$$

where *a*, *b*, and *c* are the coefficients in the lowest Kramer's doublet. The coefficients were varied to give the best least-squares fit to the observed *g* values with the relations (Kotani, 1961; Weissbluth, 1974; Huynh et al., 1978):

$$g_x = 2|(b + c)^2 - a^2|$$

$$g_y = 2|(a - c)^2 - b^2|$$

$$g_z = 2|(a - b)^2 - c^2| \quad (2)$$

with the normalization condition that[1]

$$a^2 + b^2 + c^2 = 1 \quad (3)$$

The *g* values calculated from the optimized coefficients are given in parentheses in Table I. For the most part, the *g* values calculated from eq 2 are in very good agreement with the observed *g* values.[2]

As indicated in the diagram, bands I and II in the optical spectra have been assigned to charge-transfer promotions from the two top-filled porphyrin orbitals into the iron d_{yz} orbital (Cheng et al., 1973; Stephens et al., 1976; Eaton & Hofrichter, 1981). This interpretation is based on the prediction of extended Hückel theory that the $a_{2u}(\pi)$,$a_{1u}(\pi) \rightarrow d_{yz}$ promotions are the lowest frequency charge-transfer transitions in the spectrum (Zerner et al., 1966; Eaton & Hofrichter, 1981), on the finding of a very weak natural circular dichroism to eliminate the possibility of the magnetic-dipole allowed $d_{xy} \rightarrow d_{yz}$ or $d_{xz} \rightarrow d_{yz}$ transitions, and on the observation of a large magnetic field induced circular dichroism (Cheng et al., 1973; Stephens et al., 1976).

To make the connection between the optical frequencies and the orbital parameters derived from the EPR data, we adopt the simplest theoretical model. We express the frequencies,

[1] Because the fitting program could not incorporate nonlinear constraints, the normalization condition of eq 3 was satisfied by making the substitutions $a = \cos t$, $b = \sin t \cos u$, and $c = \sin t \sin u$ in eq 2.

[2] Very similar values for Δ_1/λ and Δ_2/λ were obtained by using the analytic relations given by Taylor (1977), in which eq 2 is solved without the normalization condition.

ν_I and ν_{II}, of the charge-transfer transitions, $a_{2u}(\pi) \rightarrow d_{yz}$ and $a_{1u}(\pi) \rightarrow d_{yz}$, in terms of one-electron orbital energies as

$$\nu_I = C_{J,K} + \epsilon(d_{xy}) - \epsilon(a_{2u}) + \lambda[(\Delta_1 + \Delta_2)/\lambda]$$

$$\nu_{II} = C_{J,K} + \epsilon(d_{xy}) - \epsilon(a_{1u}) + \lambda[(\Delta_1 + \Delta_2)/\lambda] \quad (4)$$

where $C_{J,K}$ contains the Coulomb and exchange terms. Figure 2 shows a plot of ν_I and ν_{II} vs. $(\Delta_1 + \Delta_2)/\lambda$. The least-squares lines through the points have slopes of 550 ± 70 and 500 ± 80 cm^{-1} for bands I and II, respectively. These slopes are very similar to the value of $\lambda = 460$ cm^{-1} for the free ferric ion obtained from atomic spectroscopy (Dunn, 1961). The finding of a linear relation between the optical frequencies and $(\Delta_1 + \Delta_2)/\lambda$ with a slope near λ for both bands I and II indicates that the first three terms on the right-hand sides of eq 4 are approximately constant in all the complexes. The relative insensitivity of the d_{xy}, $a_{2u}(\pi)$, and $a_{1u}(\pi)$ orbital energies compared to the energy of d_{yz} is expected from qualitative considerations; d_{xy} is a nonbonding orbital, and the porphyrin π orbitals are distant from the axial ligand. In contrast, the d_{xz} and d_{yz} orbitals can participate in π bonding to the axial ligands, and their energies are therefore expected to be much more sensitive to the nature of the ligand. Iterative extended Hückel calculations are consistent with these qualitative ideas (Zerner et al., 1966; Eaton et al., 1978; Eaton & Hofrichter, 1981).

The interesting result of the linear relation expressed by eq 4, then, is that the change in charge-transfer frequency is mainly determined by the change in the iron d_{yz} orbital energy. The d_{yz} orbital is the acceptor orbital for the electron in the chemical reduction of ferricytochrome *c*, as well as in the "optical reduction" of the iron in the charge-transfer process, indicating a relation between the frequencies of the charge-transfer bands and oxidation–reduction potentials.

In order to help understand how the protein controls the oxidation–reduction potential of hemes, one would like to know the relative contributions of the axial ligands vs. the remainder of the protein. To separate these effects theoretically, it is useful to divide the addition of an electron to the ferric protein into two hypothetical steps: (i) excitation of an electron from the $a_{2u}(\pi)$ orbital into the iron d_{yz} orbital of the ferric protein and (ii) addition of an electron to the $a_{2u}(\pi)$ orbital to produce a ground-state ferrous protein. The energy of the first step is given, apart from relaxation effects, by the charge-transfer frequency. Comparison of the charge-transfer frequencies for the different proteins in Table I indicates that the energy of this step depends almost entirely on the axial ligands. The energy of the second step corresponds to the electron affinity of the excited ferric state. Variation in this energy should depend mainly on the electrostatic environment of the entire protein, since the energy of the $a_{2u}(\pi)$ orbital is found to be relatively insensitive to changes in both the axial ligands and the protein. Thus, this model suggests that the major effect of changing the axial ligands on the oxidation–reduction potential can be estimated from the change in charge-transfer frequency.

The most important biological comparison is between a cytochrome with methionine and histidine as axial ligands, as in cytochrome *c*, and one with two histidine axial ligands, such as cytochrome b_5. The oxidation–reduction potential of cytochrome *c* is 260 mV (Margalit & Schejter, 1973), while that of cytochrome b_5 is 20 mV (Kawai et al., 1963). The increase in charge-transfer frequency of 800 cm^{-1} resulting from replacement of methionine with imidazole in cytochrome *c* (Table I) corresponds to a decrease in oxidation–reduction potential of 100 mV.[3] This value is comparable to the value of 146–168 mV obtained from measurements of oxidation–reduction potentials on iron–porphyrin model compounds with imidazole/imidazole and imidazole/thioether axial ligands (Marchon et al., 1982). These results suggest that it would be worthwhile to measure the near-infrared optical frequencies of a series of low-spin cytochromes in order to assess more precisely the contribution of the axial ligands, relative to the protein environment, toward determining differences in their oxidation–reduction potentials.

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[3] One of the important environmental effects of the protein on the oxidation–reduction potential of the heme is the total net charge (George et al., 1966). In the most naive model, this effect can be estimated by the difference in free energy required to add an electron to a uniformly charged sphere immersed in water, having the radius and net charge of the protein (Schejter et al., 1982). Assuming a net charge (Z) of 5+ for ferricytochrome *c* (Margalit & Schejter, 1973) and 6– for ferricytochrome b_5 (Ozols & Strittmatter, 1969) and a 1.6-nm radius (R) for both proteins, the oxidation–reduction potential of cytochrome b_5 at zero ionic strength is calculated from the relation ΔE^0 (mV) = $18[\Delta Z/R(\text{nm})]$ (Tanford, 1961) to be 125 mV lower than that for cytochrome *c*. Thus, the total difference in oxidation–reduction potentials is calculated from the differences in charge-transfer frequencies and electrostatic work terms to be 225 mV, in excellent agreement with the observed value of 240 mV. Although this agreement may be fortuitous, it does suggest that the differences in charge-transfer frequency and net charge can account for most of the difference in oxidation–reduction potentials between cytochromes *c* and b_5.

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Resonance Raman Spectra of Blue Copper Proteins: Assignments from Normal Mode Calculations and Copper-63/Copper-65 and H_2O/D_2O Shifts for Stellacyanin and Laccase†

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ABSTRACT: Resonance Raman (RR) spectra are reported for azurin, stellacyanin, and both tree and fungal laccase; effects of type 2 Cu removal on the laccase RR spectra are noted. Normal coordinate calculations are carried out on hypothetical Cu complexes with structures related to the known active-site structure of plastocyanin and azurin by using force constants transferred from model complexes. Among the Cu–ligand stretching coordinates, only the Cu–S(Cys) stretch contributes significantly to modes in the 400-cm^{-1} region, where the strongest RR bands are found; Cu–N(His) stretching modes are expected in the 230–310-cm^{-1} range. Stellacyanin and tree laccase have been reconstituted with ^{63}Cu and ^{65}Cu, and with D_2O, to assess the Cu and imidazole motions in the RR modes. A 2-cm^{-1} D_2O/H_2O shift identifies the 273-cm^{-1} stellacyanin RR band as a Cu–N(His) mode; the extent of the shift suggests that the C2, as well as N3 imidazole, proton was replaced by D. Much smaller D_2O/H_2O shifts are seen for the strong stellacyanin or laccase bands. The two strong RR bands of stellacyanin, 347 and 385 cm^{-1}, show 1.8- and 1.5-cm^{-1} $^{63}Cu/^{65}Cu$ isotope shifts; the combined shift is that calculated for the Cu–S(Cys) stretch. It is suggested that the pair of bands arises from strong coupling between the Cu–S stretching and S–C–C bending coordinates of the bound cysteine. The average frequency is ~30 cm^{-1} lower than the average frequencies of the strong bands in laccase, azurin, and plastocyanin, consistent with the longer extended X-ray absorption fine structure derived Cu–S distance in stellacyanin (2.19 Å) than in plastocyanin or azurin (2.13 Å). The laccase $^{63}Cu/^{65}Cu$ shifts, however, 0.5 cm^{-1} or less for the strong bands at 381, 405, and 420 cm^{-1}, are much lower than those for stellacyanin and additional couplings are implicated. A variety of angle-bending modes of the coordinated ligands are expected in the 300–500-cm^{-1} region, but it is difficult to account for resonance enhancement for most of them. It is suggested that torsional motions about the cysteine S–C bond might contribute significantly to the resonance-enhanced modes.

The "blue" or type 1 site of copper proteins has long attracted a great deal of interest, because of its unusual spectroscopic properties (Malkin & Malmström, 1970; Fee, 1975; Gray & Solomon, 1981). In the past few years, the molecular and electronic structure of this site has come sharply into focus thanks to the application of a battery of physical methods, including absorption and circular dichroism (CD) spectroscopy (McMillen et al., 1974a,b; Solomon et al., 1976a,b), nuclear magnetic resonance (NMR) spectroscopy (Markley et al., 1975; Hill et al., 1976; Ugerbil et al., 1977), electron paramagnetic resonance (EPR) spectroscopy (Vanngard, 1972), spin-echo EPR spectroscopy (Mims & Peisach, 1978, 1979), electron nuclear double resonance (ENDOR) spectroscopy (Roberts et al., 1980), X-ray crystallography [Colman et al., 1978; Adman et al., 1978; Freeman, 1980 (atomic coordinates in Brookhaven Protein Data Banks); Adman & Jensen, 1981], extended X-ray absorption fine structure (EXAFS) (Tullius et al., 1978; Peisach et al., 1982), and, recently, single-crystal absorption and EPR spectroscopy (Penfield et al., 1981). The X-ray crystal structure of oxidized plastocyanin [Freeman, 1980 (atomic coordinates in Brookhaven Protein Data Banks); Figure 1] and azurin (Adman et al., 1978) shows the copper ion to be coordinated to a cysteine sulfur and two hystidyl imidazole ligands, and also to a distant (2.9 Å) methionine sulfur atom, in a highly distorted tetrahedral complex. Stellacyanin has similar electronic properties but lacks methionine. The EXAFS analysis (Peisach et al., 1982) confirms the presence of two close N ligands and a close S ligand, with

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