Spectral Splitting in the α ($Q_{0,0}$) Absorption Band of Ferrous Cytochrome *c* and Other Heme Proteins†

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ABSTRACT: The α or $Q_{0,0}$ absorption band of horse iron(II) cytochrome *c* splits and shifts to the blue as temperature decreases over the temperature range of 290−10 K. At room temperature, its maximum is at 18 150 cm^{-1} and the spectral width is 273 cm^{-1}, whereas at 10 K, the two bands of the $Q_{0,0}$ transition occur at 18 364 and 18 253 cm^{-1} and the width of the lowest-energy band is 96 cm^{-1}. Temperature dependent splitting also occurs for zinc cytochrome *c*, a derivative in which Fe has been replaced by Zn; at 10 K, the peaks in the $Q_{0,0}$ band region occur at 17 106 and 16 996 cm^{-1}. The peak positions are independent of the cryosolvent (aqueous ethylene glycol or glycerol mixtures). The splitting of the $Q_{0,0}$ band seen in the protein (∼110 cm^{-1} for iron and zinc cytochrome *c*) is comparable to the crystal field splitting observed for metalloporphyrins in mixed crystals. In contrast, the $Q_{0,0}$ band of zinc coproporphyrin III in a glassy solvent (dimethylformamide/ethylene glycol) or in poly(vinyl chloride) shows a blue shift with temperature decrease but no evidence of $Q_{0,0}$ splitting. Available spectral data show that the $Q_{0,0}$ band is composed of two nearly degenerate electronic transitions and the split is due to the asymmetry in the heme pocket of the protein that arises from the surrounding polypeptide chain. This asymmetry results in the stabilization of one form of the excited state over the other, according to a Jahn−Teller mechanism.

Heme proteins are involved in a myriad of cellular processes, including electron transfer, transport of diatomic molecules, cellular regulation, and hydroxylation reactions. How the unique properties of hemes are modified by interactions with the polypeptide chain leading to specificity of function presents itself as an intriguing biological problem.

The spectral properties of heme proteins and model compounds, arising from porphyrin transitions, have been thoroughly reviewed (Adar, 1978; Owens & O'Connor, 1988). The four-orbital model proposed for the interpretation of metalloporphyrin spectra (Gouterman, 1978) describes transitions allowed under D_{4h} symmetry, between the two highest occupied molecular orbitals, $a_{1u}(\pi)$ and $a_{2u}(\pi)$, and the two degenerate lowest unoccupied orbitals, $e_g(\pi^*)$. The degenerate nature of these latter orbitals results in strong electron interaction between them and accounts for the relatively pure $\pi-\pi^*$ transition, the B or Soret band, in which the transition dipoles add up and for the weak $Q_{0,0}$ or α band in which the dipoles almost cancel. An additional band, the $Q_{1,0}$ or β, is attributed to vibronic coupling.

Most theoretical calculations have been performed on square planar D_{4h} models. Asymmetric substituents on the porphyrin ring, metal movement out of the porphyrin plane, and insertion of the heme into a protein matrix effectively lower this symmetry to C_{4h} (Adar, 1978) and in some cases even to C_s, where the only remaining element of symmetry is the planarity of the porphyrin macrocycle (Valance & Strekas, 1982). In spite of these symmetry-lowering factors, some heme proteins do manage to maintain an effective 4-fold rotational axis (Eaton & Hochstrasser, 1967). In ferrous cytochrome *c* (cyt *c*),[1] the heme is covalently bound to the protein via thioether linkages between the porphyrin vinyl side chains and cysteine residues. Two strong field ligands, histidine 18 and methionine 80, provide axial coordination to a low-spin iron(II) ($S = 0$) (Makinen & Churg, 1983). Since the six d electrons fill the d_{xz}, d_{yz}, and d_{xy} orbitals, metal−porphyrin interactions are considered minimal. These orbitals, however, represent a possible path for the decay of the excited state. If the $Q_{0,0}$ band is assigned as an $a_{2u}(\pi)-e_g(\pi^*)$ transition, a d electron can then fall back into the a_{2u} orbital, leaving a hole for the e_g (π^*) electron to decay to. Such a pathway is obviously not available for closed-shell metal porphyrins (Adar, 1978).

It has been known for almost half a century that the $Q_{0,0}$ transition absorption bands of some heme proteins show spectral splitting or band asymmetry at room temperature or as the temperature is decreased (Keilin & Hartree, 1949; Wilson, 1967; Hagihara et al., 1974; Wagner & Kassner, 1975; Champion et al., 1976; Friedman et al., 1977; Cowan & Gray, 1988, 1989a; Ahn et al., 1995). Various interpretations have been proposed to account for *Q* band splitting which is theoretically predicted for any mechanism capable of lifting the $e_g(\pi^*)$ degeneracy (Gouterman, 1978). In a study of different cytochromes, Wagner and Kassner (1975) proposed that coordination of the proximal histidine and its effect on the heme pocket environment could lift the degeneracy of the *Q* band. Cowan and Gray (1989b) argued that $Q_{0,0}$ splitting depends on distinct axial ligation arising from two porphyrin orientational isomers (La Mar et al., 1984). In addition to reporting *Q* band splittings in cytochromes, Hagihara et al. (1974) noted a shift of the

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[1] cyt, cytochrome; JT, Jahn−Teller; FWHM, full width at half-maximum.

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absorption maxima and intensification of extinction as temperature was lowered. In studying the temperature dependence of Zn-substituted myoglobin, Ahn et al. (1995) came to similar conclusions on the *Q* band splitting; it is due to the lifting of the degeneracy between the porphyrin and its environment. This was also previously argued for Raman data of heme proteins and metalloporphyrins (Friedman et al., 1977; Shelnutt et al., 1977). The same phenomenon has been observed in metalloporphyrin coordination compounds in crystalline host matrices (Canters et al., 1976; Kim & Bohandy, 1977; Jansen et al., 1978; Platenkamp & Canters, 1981). The mechanisms assumed to be active in lifting the $e_g(\pi^*)$ orbital degeneracy have been attributed to Jahn−Teller type distortions (Canters et al., 1972; Platenkamp & Canters, 1981), to crystal field interactions (Kim & Bohandy, 1977), and to the various sites produced by the alignment of electric field axes at different angles with respect to the molecular axes of the porphyrin (Leenstra, 1979).

The question of the mechanism(s) responsible for the $Q_{0,0}$ band spectral behavior, i.e., its splitting, its shift with temperature, and its change in extinction coefficient, is of importance in understanding how the polypeptide chain modulates the spectral and biological properties of the porphyrin macrocycle. It also centers on a question which we find particularly interesting. Do we need to invoke the existence of more than one protein conformation to account for them? In this paper, we examine the optical absorption spectrum of horse cyt *c*, a small monomeric mitochondrial protein. The iron in Fe cyt *c* can undergo ligand changes that may alter the spectrum (Brautigan et al., 1977). Furthermore, although the spin state is $S = 0$ for Fe(II) cyt *c*, it has low-lying d orbitals which produce a shortening of the excited singlet lifetime (∼0.2 ps) (Champion & Lange, 1980). We, therefore, also examine the spectrum of cyt *c* in which the metal is replaced with the closed-shell metal ion, Zn(II). Zn cyt *c* has protein folding nearly identical to that of the native Fe(II) cytochrome (Anni et al., 1995), but unlike Fe(II) cyt *c*, it has a long-lived singlet and triplet excited states (Vanderkooi et al., 1976). Spectral broadening in Zn cyt *c* is therefore due to inhomogeneity, rather than to lifetime or other broadening mechanisms (Vanderkooi et al., 1985; Logovinsky et al., 1993). Finally, zinc coproporphyrin III in a glassy and polymer matrix is similarly investigated and compared with the metalloporphyrins in the protein matrix.

MATERIALS AND METHODS

Horse heart cyt *c* and poly(vinyl chloride) were obtained from Sigma Chemical Co. (St. Louis, MO); it was reduced to Fe(II) cyt *c* by dithionite addition before measurement. Zn cyt *c* was prepared from horse Fe cyt *c* as previously described (Vanderkooi et al., 1976).

To prepare the coproporphyrin polymer films, poly(vinyl chloride) (MW of 50000−100000) and zinc coproporphyrin III (Midcentury Chem., Posen, IL) were dissolved in benzene and the solutions mixed. The viscous solution was spin coated on a glass plate and then allowed to evaporate for ∼48 h in the dark. The film was peeled off and mounted in the cyrostat sample holder.

For all absorption measurements, the sample concentration was 1−8 mM. A Bruker IFS 66 FTIR spectrometer (Bruker Instruments, Inc., Billerica, MA) equipped with a tungsten source, a Si beam splitter, and a silicon diode detector for the high-resolution optical measurements. The path length of the cell was 0.05 mm, and sapphire windows were used. The temperature of the sample was controlled with a circulation bath and a thermoelectric module from ambient until −8 °C. For temperatures below that, a top-loading APD closed cycle Helitran cryostat (Advanced Research Systems, Allentown, PA) was used. The temperature was measured with a silicon diode near the sample. In this cryostat, the sample chamber is filled with He gas at atmospheric pressure so that temperature variations between the sample and the measuring point can be expected to be negligible. The cryostat and sample holder windows were of sapphire.

A Norton−Beer apodization function was used to correct the spectral response, and the spectral resolution was 0.5−2 cm^{-1}. Spectral bands were fit to Lorentzian or Gaussian functions using PEAK Fit (Jandel Co.) or OPUS (Bruker Instruments, Inc.). A Levenberg−Marquardt algorithm was used for the minimization.

Phosphorescence lifetimes were measured using the equipment, data acquisition, and analysis programs that were described previously (Green et al., 1988). The sample was held in place by a cold-finger type optical dewar. Temperature regulation was achieved by passing the evaporated nitrogen from a liquid nitrogen dewar through the optical dewar and the sample temperature adjusted by changing the flow rate. A thermocouple at the sample continuously monitored the temperature. The samples were deoxygenated as previously described (Horie & Vanderkooi, 1981).

RESULTS

Temperature Dependence of the Absorption Spectrum of Fe(II) cyt c. The absorption spectra of Fe(II) cyt *c* in 50% ethylene glycol were taken at different temperatures from 290 to 10 K as shown in Figure 1. At 270 K, the absorption of the $Q_{0,0}$ transition is centered at 18 185 cm^{-1}, close to the literature value (Margoliash & Frohwirt, 1959), and the width of this transition is 273 cm^{-1}. As temperature is decreased, there is a shift in the absorption of the $Q_{0,0}$ band to a higher frequency. By ∼170−180 K, a noticeable split in the $Q_{0,0}$ transition occurs and the β band (i.e., the $Q_{0,1}$ or "Q_{vib}" transition, composed of many vibrational lines between 19 000 and 20 000 cm^{-1}) also begins to show resolution. As the temperature is further decreased, the lines continue to sharpen and shift. It can be noted also that the overall intensity of the $Q_{0,0}$ band increases as temperature decreases; integration of the $Q_{0,0}$ band region showed that the intensity increased by about 1.8 in going from 200 to 10 K.

To emphasize the temperature dependence, the $Q_{0,0}$ band region of the spectrum of Fe(II) cyt *c* is expanded in Figure 2. The peak position at room temperature, 18 185 cm^{-1} (see Figure 1), shifts to 18 188 cm^{-1} at 240 K, indicated in Figure 2. At 10 K, the center of the lowest absorption peak in the $Q_{0,0}$ transition is at 18 253 cm^{-1} and the next transition is at 18 364 cm^{-1}. No isosbestic point is seen in these spectra as temperature is changed, and the relative population and the frequency difference between the two peaks do not appear to change with temperature.

During the temperature decrease, the sample visually appeared to be a glass. The spectral widths and positions reversed when the temperature was cycled between 12 and

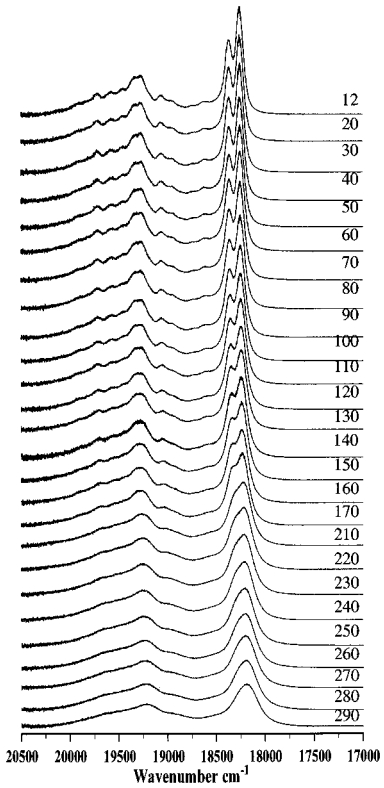

FIGURE 1: Absorption spectrum of Fe(II) cyt *c* in 10 mM phosphate buffer (pH 7.0) and 50% ethylene glycol. The temperatures in kelvin are indicated on the figure. The temperature was changed from high temperatures to low; the time to change the temperature by 10 K was about 10 min, and the temperature was held constant for about 5 min during the time it took to acquire the spectrum. The peak maximum at 290 K is indicated by the line.

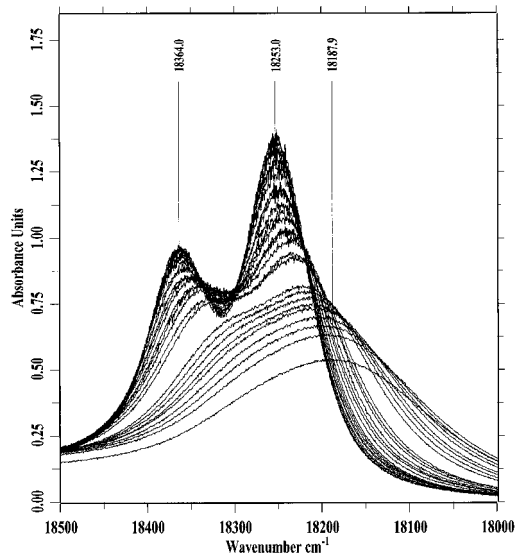

FIGURE 2: Data from Figure 1 plotted on an expanded scale. Temperatures range from 290 to 12 K and can be obtained from Figure 1. Peak positions at 12 and 290 K are indicated.

170 K. Above 170 K, with warming, the sample sometimes becomes scattering. For this, the solvent composition plays a role, as 50% ethylene glycol/buffer can crystallize, and the sample is no longer glassy.

Absorption of Zn cyt c. In order to examine whether the spectral shifts for native Fe(II) cyt *c* could be attributed to changes in ligation to the iron, we also examined the absorption spectra of Zn(II) cyt *c*. Its absorption spectra in

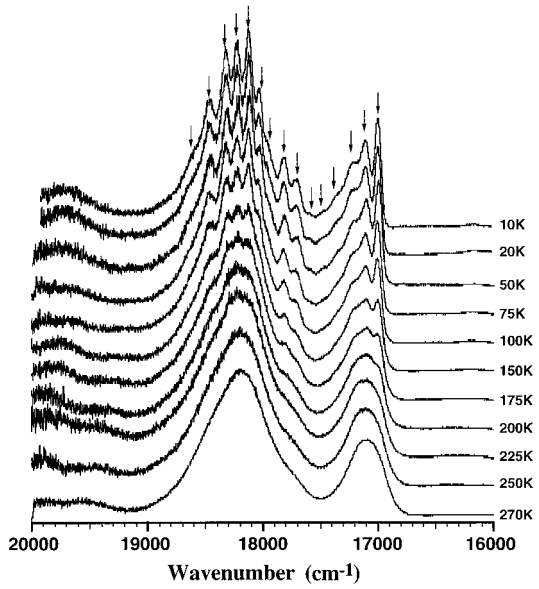

FIGURE 3: Absorption spectrum of Zn cyt *c* in 10 mM phosphate buffer (pH 7.0) and 50% ethylene glycol. Temperatures are indicated on the figure. Temperature was regulated as described in Figure 1.

1/1 ethylene glycol/water at different temperatures are shown in Figure 3. The overall temperature profiles are very similar to that of the native Fe(II) cyt *c*. Again, there is a blue shift and increased resolution in the $Q_{0,0}$ transition as temperature is lowered, although the shift in the spectrum is not as pronounced as for Fe(II) cyt *c*. The center of the $Q_{0,0}$ absorption at room temperature is 17 080 cm^{-1}. At 10 K, the absorption maxima of the $Q_{0,0}$ band components were at 16 996 and 17 106 cm^{-1} and bands at a higher frequency, attributed to vibronic lines, are indicated by arrows. Again, the overall intensity of the transition increased as temperature was lowered.

Since large motions in the protein, especially at the protein surface, will be affected by solvent viscosity, we repeated the experiment with Zn cyt *c* dissolved in 80% glycerol and 10 mM phosphate aqueous buffer. The viscosity of the glycerol is much higher than that of ethylene glycol (cf. below). The spectra at different temperatures are shown in Figure 4. The relative peak heights are different, but the overall positions and temperature dependence are very much the same for the two solvents. Figure 5 shows the $Q_{0,0}$ band region at various temperatures, and the results can be compared to those for Fe(II) cyt *c*, shown in Figure 2.

The absorption spectrum of the protein in a polycrystalline matrix was also examined. The aqueous buffer without cryosolvent is crystalline and is highly light-scattering at low temperatures. As seen in Figure 6, the spectra of Zn cyt *c* are not resolved at all measured temperatures but begin to show some fine structure at temperatures below 100 K.

Deconvolution of the Spectra. Electronic spectral bands of chromophores in protein matrix are complex, and simple functions may not be appropriate to fit the peaks of absorption transitions (Austin & Erramilli, 1995). If the peak widths were solely determined by lifetime without various coupling mechanisms, a Lorentzian function might be used; if the width is due to fluctuations that are of a large number and are harmonic and isotropic, a Gaussian would pertain. However, a protein would be expected to exhibit correlated, not random, motions. The results of fitting for Fe(II) are shown in Figure 7 and Table 1. The fitting allowed the

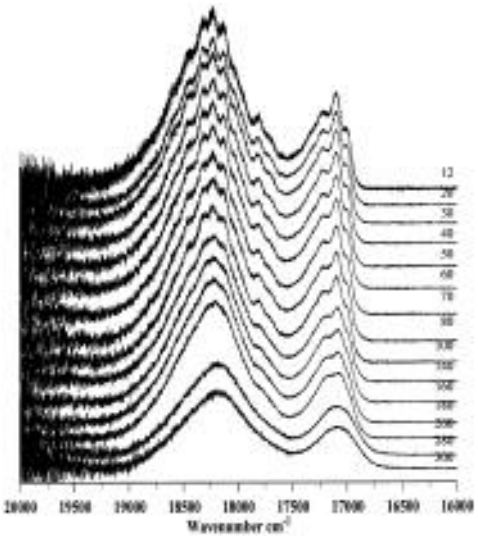

FIGURE 4: Absorption spectrum of Zn cyt *c* in 10 mM phosphate buffer (pH 7.0) and 80% glycerol. Temperatures are indicated on the figure, and the maximum is at 305 K. Temperature was changed as described in Figure 1.

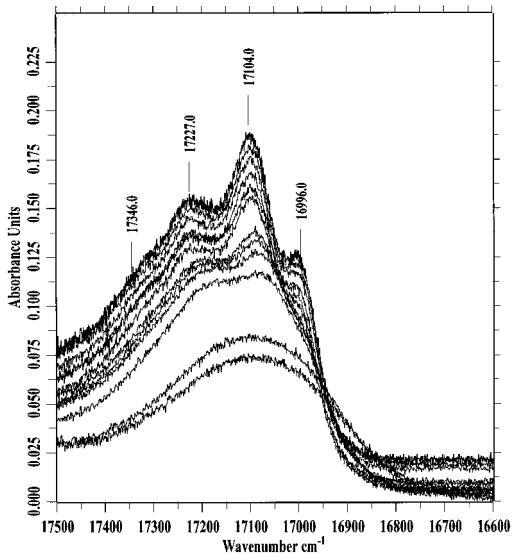

FIGURE 5: Data from Figure 4 plotted on an expanded scale. Temperatures range from 305 to 12 K and can be obtained from Figure 4. Peak positions are given for 12 K

detection of a duplication of peaks in the spectra of Fe(II) cyt *c*. The lowest peaks occur at 18 253 and 18 364 cm^{-1}, separated by ~110 cm^{-1}; as discussed later, these are identified as electronic origins. Two pairs of peaks also separated by ~110 cm^{-1} can be found as we move into the vibronic region; they are at 18 617 and 18 735.3 and at 18 952 and 19 063 cm^{-1}. This constant separation of ~110 cm^{-1} supports the idea of splitting in the electronic transition. In the $Q_{0,1}$ region, which is composed of many vibrational bands, duplication of lines could not be discerned, probably because this area is so congested.

Temperature Dependence of the Absorption Spectrum of Zinc Coproporphyrin III. Recent work has established that the $Q_{0,0}$ absorption spectrum of zinc protoporphyrin IX in aqueous glass has no splitting at 1.4 K (Köhler et al., 1996). To confirm the behavior of the $Q_{0,0}$ band, in a glass, we acquired the absorption spectrum of zinc coproporphyrin III in dimethylformamide/glycerol glass (Figure 8). This is a porphyrin which has the same chromophoric ring structure

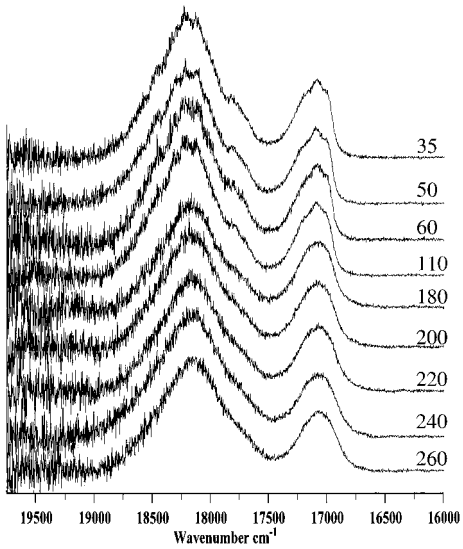

FIGURE 6: Absorption spectrum of Zn cyt *c* in 10 mM phosphate buffer (pH 7.0). Temperatures, in kelvin, are indicated on the figure.

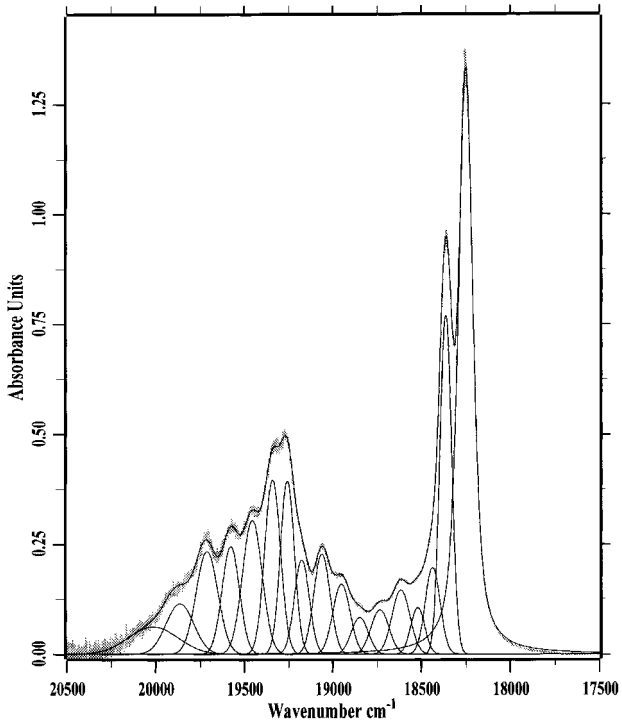

FIGURE 7: Deconvolution of the absorption spectrum of Fe(II) cyt *c* in 10 mM phosphate buffer (pH 7.0) and 50% ethylene glycol. The fitting parameters are given in Table 1. Temperature = 10 K.

as the porphyrin found in cyt *c* but is more soluble in polar solvents due to the additional two carboxylate groups. The absorption spectrum of the same chromophore in poly(vinyl chloride) film is shown in Figure 9. Poly(vinyl chloride) represents a polymer, but without the defined secondary and tertiary structure or charge distribution that a protein has. For both matrices, as temperature is lowered, the $Q_{0,0}$ band shows a continuous shift to higher frequency without the evidence of splitting.

*Spectra and Phosphorescence Lifetime of Zinc Porphyrins in Glycerol and Ethylene Glyco*l. Protein fluctuations are influenced by surrounding solvent motions, and two solvents were used to examine whether the viscosity during cooling influenced the spectra (Figures 3 and 4). An independent measure of the solvent motion was obtained by comparing

Table 1: Fit Parameters to the Absorption Spectrum of Fe(II) cyt *c* at 12 K

position	intensity	width	shape
18 253	1.33	96	62% Lorentz
18 364	0.76	75	Gauss
18 438	0.20	96	Gauss
18 522	0.10	90	Gauss
18 617	0.14	115	Gauss
18 735	0.10	115	Gauss
18 849	0.08	110	Gauss
18 952	0.16	111	Gauss
19 063	0.23	105	Gauss
19 176	0.21	97	Gauss
19 257	0.39	90	Gauss
19 341	0.39	102	Gauss
19 455	0.30	128	Gauss
19 577	0.24	113	Gauss
19 711	0.23	142	Gauss
19 864	0.11	180	Gauss
20 014	0.06	323	Gauss

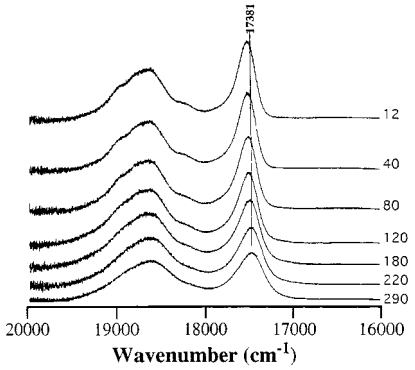

FIGURE 8: Absorption spectrum of zinc coproporphyrin in 50/50 (v/v) dimethylformamide/ethylene glycol. Temperatures are indicated on the figure. The peak position at 290 K is indicated.

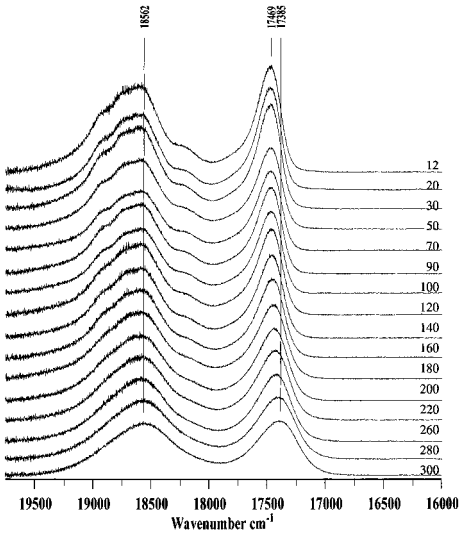

FIGURE 9: Absorption spectrum of zinc coproporphyrin in poly(vinyl chloride) film. Temperatures are indicated on the figure. Peak positions at 300 and 12 K are indicated.

the relaxation time of the phosphorescence in the two solvents. The phosphorescence decay profile and the emission spectra are shown in Figure 10. The phosphorescence lifetime of zinc coproporphyrin III dissolved in solvent is 65 ms at 77 K. At 77 K, the lifetime of Zn cyt *c* is lower than that of zinc coproporphyin; this has been observed previously, and we considered that the thioether linkages to the polypeptide chain or the ligated methionine (Anni et al., 1995) are possible phosphorescence quenchers.

In Figure 11, the lifetime of the phosphorescence of Zn cyt *c* and zinc coproporphyrin is given as a function of temperature. The ethylene glycol sample "softened", as indicated by the change in phosphorescence lifetime as temperature changes in the range of >100 K, whereas the glycerol sample showed a marked decrease in the range >250 K. The different temperature dependence is consistent with the known temperature dependence of the viscosity of the two samples. At 20 °C, 80% glycerol has a viscosity of about 60 cP, i.e., ~60 times more viscous than water, whereas the viscosity of ethylene glycol at 50% is only 4 times that of water (Timmermans, 1960). At 0 °C, the viscosity of 80% glycerol goes to 255 cP.

In contrast to that of zinc coproporphyrin III, the phosphorescence lifetime of the Zn cyt *c* remains constant in glycerol for temperatures between 77 and 200 K and decreases slowly in 50% ethylene glycol at temperatures above 200 K.

DISCUSSION

These basic experimental observations are presented. With decreasing temperature, the absorption spectra of Fe(II) and Zn cyt *c* shift to the blue, the $Q_{0,0}$ band splits, the lines in the visible absorption region sharpen, and the extinction coefficient increases.

Table 2 presents *Q* band splittings observed for various heme proteins. Fe(II) systems show "true" splittings, in that two *Q* components are observed when splitting occurs. The low-temperature absorption spectra of Zn cyt *c* show more than two *Q* components (Figures 3−6). A recent experiment measuring hole widths across the two lowest-frequency peaks of the inhomogeneous split band has shown that the hole is constant with a width of 300 MHz (Köhler et al., 1996). This is equivalent to a relaxation time of ≥1 ns, showing that these two absorption bands also arise from electronic origins in the metal-substituted heme protein. In contrast, broad holes burnt in the higher-energy bands indicate that these are due to vibrational bands. We can thus conclude that Zn cyt *c*, like its parent, Fe(II) cyt *c*, exhibits $Q_{0,0}$ splitting. Two explanations are considered to account for two electronic origins in these systems.

(1) The observed splitting may arise from the chromophore itself; i.e., it is a homogeneous effect, and the absorption spectra of all molecules in the sample are split. This behavior could arise by a crystal-field-induced Jahn−Teller (JT) effect; the splitting occurs because the protein exerts an asymmetric electric or crystal field. A diagram of crystal-field-induced JT splitting and interactions is explained in Figure 12. The JT theorem states that the orbital degeneracy and stability of the nuclear configuration are incompatible except for molecules in which all the atoms lie in a straight line (Jahn & Teller, 1937). Hougen, in particular, showed that molecules with 4-fold symmetry such as metal porphyrins are JT unstable and coupling along a single normal coordinate lowers the potential energy wells of the two orbital components, $|x\rangle$ and $|y\rangle$, associated with an E_u state with a concomitant shift in opposite directions along the normal coordinate (Hougen, 1964). These two orbital components in free space are energy equivalent since the JT effect does not lift the degeneracy of the two vibronic states. Local symmetry breaking due to axial ligation, porphyrin lateral

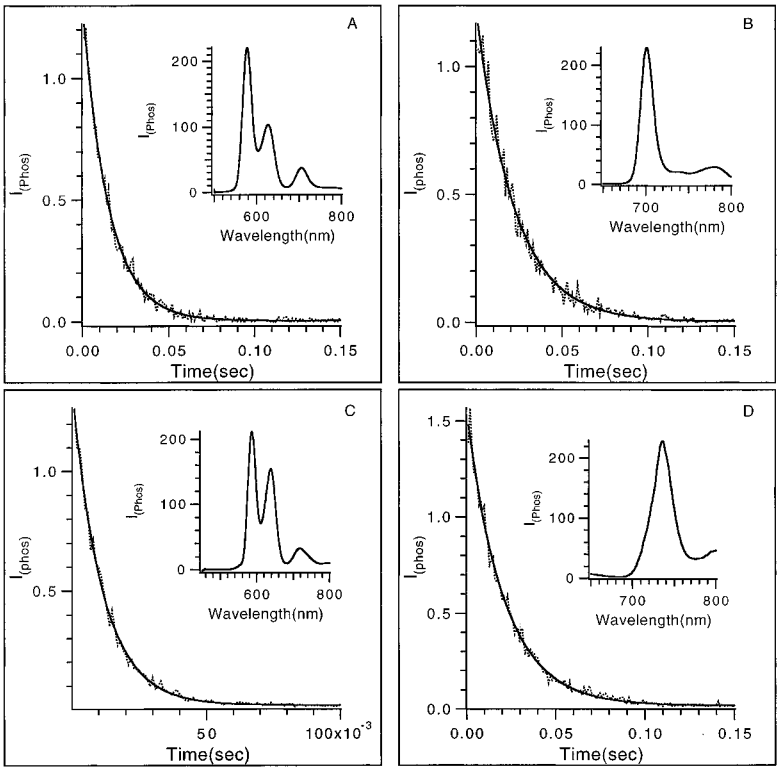

FIGURE 10: Phosphorescence decay profiles and spectra (inset). Zinc coproporphyrin was excited at 423 nm and emission detected at 704 nm maintained at 20 °C (A) and 77 K (B); Zn cyt *c* was excited at 423 nm and emission detected at 736 nm maintained at 20 °C (C) and 77 K (D). Solvent was 50% ethylene glycol and 10 mM phosphate buffer (pH 7.0). The solid line is an exponential fit to the data with a lifetime of 22.0, 65, 11.1, and 20.8 ms for A−D, respectively. Insets show the emission spectra obtained with 423 nm excitation taken 1 ms after the flash and with a 9 ms gate time.

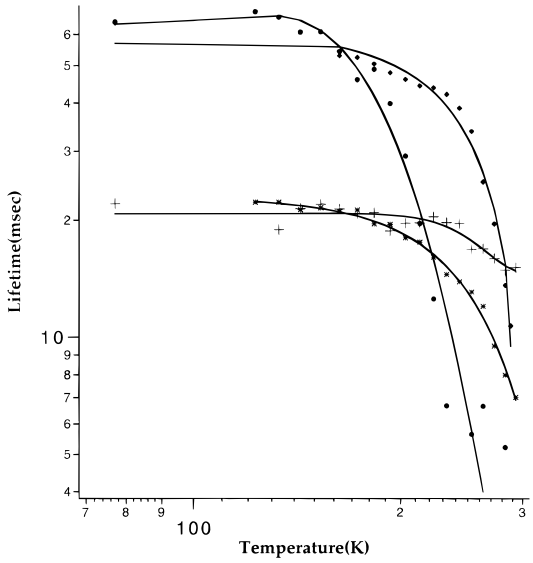

FIGURE 11: Temperature dependence of phosphorescence lifetime: zinc coproporphyrin IX in 80% glycerol (◆), zinc coproporphyrin in 50% ethylene glycol (●), Zn cyt *c* in 80% glycerol (+), and Zn cyt *c* in 50% ethylene glycol (*).

substituents, or matrix electrostatic effects can lift the vibronic degeneracy and selectively stabilize one vibronic component (Canters et al., 1972, 1973).

(2) An alternate possibility is that the origin of the split is due to "conformational substates"; one population shows its origin at 18 364 and the other at 18 252 cm^{-1} for Fe(II) cyt *c*. Proteins display structural fluctuations (Cooper, 1984). It follows that at any instant there will be a distribution of properties. Results from a variety of approaches have demonstrated that for heme proteins each subconformation of the protein has fundamentally different chemical and physical properties (Austin et al., 1975; Karplus & McCammon, 1986; Frauenfelder et al., 1988; Goldanskii & Krupyanskii, 1989). One can imagine that as temperature is lowered and viscosity increases some motions become damped. In this model, one would expect a distribution of mean locations of the atoms around the chromophore. This is experimentally verified by X-ray diffraction for myoglobin (Frauenfelder et al., 1979). However, conformational substates may become frozen in as the temperature becomes too low to overcome the energy barrier between the subconformational states and are energetically trapped into local minima (Elber & Karplus, 1987; Frauenfelder et al., 1991) or kinetically trapped by the high (approaching infinity) viscosity of the solvent (Ansari et al., 1992). This idea of a hierarchy of substates is substantiated by the observation that narrow band "holes" burned into the inhomogeneous optical band of metal-free and metal-substituted hemoproteins broaden in a stepwise manner as temperature is changed (Köhler et al., 1988; Zollfrank et al., 1991b; Gafert & Friedrich, 1995; Köhler et al., 1996), as well as the observation of more than one frequency for CO infrared absorption in CO−myoglobin (Ormos et al., 1988, 1990).

As described below, we believe that the split in the $Q_{0,0}$ absorption band can be explained on the basis of the first explanation, without the need to invoke the existence of two conformations for the protein.

Spectral Splitting of Porphyrins. Numerous optical and magnetic resonance studies of metalloporphyrin-photoexcited triplet states in crystalline, glassy, and protein systems, as well as theoretical work, have firmly established the JT

Table 2: $Q_{0,0}$ Band Splittings Observed for Metalloporphyrins in Proteins and Crystals[a]

	source/matrix	$Q_{0,0}$, cm^{-1}	δQ, cm^{-1}	T, K	ref
proteins					
Fe(II) cyt *c*	horse	18 253	111	10	this work
		18 364			Champion et al., 1976; Friedman et al., 1977
Zn cyt *c*	horse	16 996	110	10	this work
		17 106			
Zn cyt *c**	horse		180		Angiolillo & Vanderkooi, 1995
Fe(II) cyt *c*	yeast		no split	4.2	Hagihara et al., 1974
Fe(II) cyt *c*	pigeon	18 248	67	9	Hagihara et al., 1974
		18 315			
Fe(II) cyt *c*	*Candida brusei*		no split	77	Wagner & Kassner, 1975
Fe(II) cyt c_1	beef	18 051	131	9	Hagihara et al., 1974
		18 182			
Fe(II) cyt c_2	*R. rubrum*	18 215	100	77	Wagner & Kassner, 1975
		18 315			
Fe(II) cyt c_3	*C. ethylicium*	18 083	165	77	Wagner & Kassner, 1975
		18 248			
Fe(II)Mb§	sperm whale	17 700	350	~40	Keilin, 1966
nicotinate		18 050		300	
Fe(II)MbCN	horse	17 513	249	100	Reddy et al., 1996
		17 762		250	
MgMPMb	sperm whale	17 123	329	300	Cowan & Gray, 1989a
		17 452			
MgPPMb	sperm whale	16 745	317	300	Cowan & Gray, 1989a
		17 062			
MgPPMb	horse	16 716	157	10	Kaposi et al., 1993
		16 873			
SnMPMb	sperm whale	17 476	170	300	Cowan & Gray, 1989a
		17 646			
SnPPMb	sperm whale	16 978	329	300	Cowan & Gray, 1989a
		17 307			
ZnPPMb	sperm whale	16 850	270	4	Ahn et al., 1993
		17 180			
Fe(II) cyt b_5	calf	17 680	196	77	Adar et al., 1976
		17 876			
	calf	17 680	200	6	Friedman et al., 1977
		17 880			
Fe(II) cyt *b*	beef		no split		Hagihara et al., 1974
Fe(II) cyt b_{562}	Escherichia coli	17 873	80	77	Wagner & Kassner, 1975
		17 953			
crystals					
zinc porphin	*n*-octane	17 961	109	4.2	Canters et al., 1972
		18 070			
MgTBP	*n*-octane	15 962	30	4.2	Platenkamp & Canters, 1981
		15 992			
zinc porphin	anthracene	17 700	80	4.2	Bohandy & Kim, 1979
		17 620			
zinc porphin	triphenylene§ I**	17 612	111	4.2	Kim & Bohandy, 1977
		17 723			
	II	17 584	273		
		17 857			
	III	17 540	285		
		17 825			

[a] Abbreviations: Mb, myoglobin; MP, mesoporphyrin IX; PP, protoporphyrin IX; TBP, tetrabenzoporphine. Measurements were taken by absorption except for that for MgPPMb which was done by fluoresecence line narrowing, and Zn cyt *c** which was the triplet state was measured by EPR; ** numerals refer to sites within the crystal.

effects (Canters et al., 1972, 1973; Hoffman, 1975; Kielman-van Luijt et al., 1976; Canters & van der Waals, 1978; Hoffman & Ratner, 1978; Canters, 1981; Angiolillo & Vanderkooi, 1995; Angiolillo et al., 1995). For Zn cyt *c*, the maximum crystal-field-induced JT splitting in the triplet state was estimated to be 180 cm^{-1} (Angiolillo & Vanderkooi, 1995). In contrast, metalloporphyrin model compounds in disordered glassy matrices reveal in some cases a crystal-field JT splitting less than 5 cm^{-1} (Angiolillo & Vanderkooi, 1995), indicating less symmetry breaking in an isotropic glassy matrix.

Evidence for the reduction of symmetry in the singlet state comes also from optical studies. In Stark-effect experiments, the low-frequency range of the inhomogeneous band for Zn cyt *c* showed a definite dipole moment, verifying the loss of strict D_{4h} symmetry. Electrostatic calculations on this protein system corroborate that the protein matrix exerts an asymmetric field on the heme (Köhler et al., 1996). However, holes burnt into the higher-energy band at 1.7 K are also narrow, implying that, if there are two excited energy levels, their interconversion rates are slow. The decay of anisotropy of magnesium tetraphenylporphyrin occurs with a time constant of 210 and 1.6 ps at room temperature (Galli et al., 1993), but these times are influenced by the solvent fluctuations, which will not play a role at the cryogenic temperatures. Therefore, "slow" interconversion between the states is not ruled out. Finally, the magnetic circular dichoism spectra of zinc and magnesium coproporphyrins

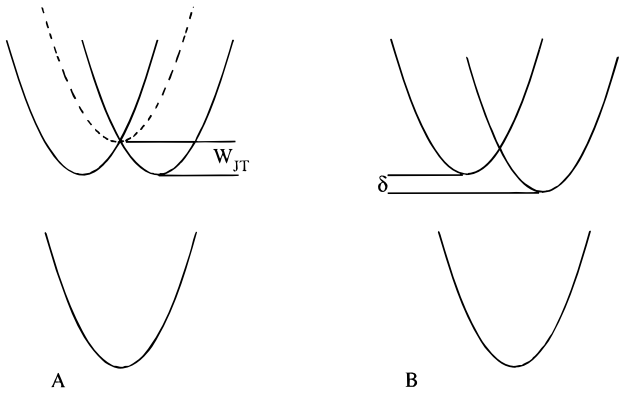

FIGURE 12: Schematic of the nuclear potential energy. (A) The dashed line represents the free molecule without vibronic coupling. The solid lines represents the molecule with vibronic coupling. The energy W_{JT} represents the stabilization due to the JT effect. (B) The case when the molecule is embedded in a host. One configuration is stabilized by the asymmetry of the host environment, and a splitting δ is observed in the origin transition. This scheme has been adapted from Canters et al. (1976).

(Sutherland, 1978) and zinc porphyrins (Mack & Stillman, 1995) showed sharpening, consistent with reduction of symmetry of the excited state.

Splitting in Heme Proteins. Table 2 summarizes the splitting observed in the $Q_{0,0}$ band of the optical spectra of porphyrins in proteins and crystals.

The splitting for the Zn cyt *c* is ~110 cm^{-1}, less than the value of 180 cm^{-1} found for the JT splitting of Zn cyt *c* in the triplet state (Angiolillo & Vanderkooi, 1995). The nature of the S_0-T_1 transition in porphyrins is predominantly a pure $\pi-\pi^*$ transition and hence susceptible to JT-related phenomena. The S_0-S_1 transition, because of the increased extent of configuration interaction, is predicted to demonstrate smaller JT splitting (Bersuker & Stavrov, 1988). The splitting magnitude of Zn cyt *c* is in the same range that is seen in model systems for zinc porphyrin singlet states in crystalline matrices. For example, the splitting for zinc porphin in an *n*-octane crystal is at 109 cm^{-1} (Canters et al., 1972). The splitting of zinc porphin in anthracence is at 79 cm^{-1} (Bohandy & Kim, 1979) and of zinc porphin in triphenylene at 111, 273, and 283 cm^{-1}, the latter values depending upon the site within the crystal (Kim & Bohandy, 1977).

An interesting point of the data in Table 2 is that closely related proteins show different splittings. The splitting did not vary significantly with the metal center for horse cyt *c*; both Zn and Fe showed the same splitting. However, yeast and *C. brusei* cyt *c* showed no detectable splitting, whereas the pigeon cytochrome showed an intermediate value.

Other heme proteins also show splitting in the $Q_{0,0}$ band, and data for myoglobin are included in the table. Keilen observed the splitting of the *Q* band at room temperature in nitrogenous base-ligated forms of myoglobin (Keilin, 1966). She concluded that these were electronic in origin since the Soret peak was not split. Reddy et al. (1996) showed that the cyanide complex of iron(II) myoglobin has a splitting on the order of 249 cm^{-1}. CN dissociates with time from myoglobin with a single-exponential function. The spectra of the bound and partially dissociated protein show clear isosbestic points. This is strong evidence that only one conformation is present since the kinetics and spectra of two conformations would differ. A similar split in the absorption spectrum of metal−myoglobin was attributed to a 180° flip of the heme (Cowan & Gray, 1989a), which is known to occur (La Mar et al., 1984). This possibility is eliminated in cyt *c* because the heme periphery is tethered in two places to the polypeptide chain and has also been discounted in ligated forms of myoglobin (DiFeo & Addison, 1991).

Finally, a striking feature of these absorption spectra is that there appear to be two, not more, electronic origins. If one were to imagine that the protein folded into a variety of conformations with well-defined energy wells, the many possible degrees of freedom for a protein would allow for muliple subconformations. The CO stretch of myoglobin, for instance, shows three or four bands (cf. above). Additionally, the value of the split is constant with temperature. This can be seen in Figures 1−5 and is especially evident in Figure 2. The split in cytochrome b_5 also does not change with temperature (196 cm^{-1} at 77 K and 200 cm^{-1} at 6 K, Table 2).

At this point, we can ask what can produce asymmetry. The heme in metalloprophyrins is not planar; it exhibits an out-of-plane distortion that is primarily of the "ruffling" type (Munro et al., 1992). This distortion is conserved in various *c* type cytochromes (Hobbs & Shelnutt, 1995) but cannot produce an in-plane distortion that would lift the degeneracy. We must then look for contributions to the ruffling distortion that would lead to a type of in-plane distortion capable of splitting the *Q* band. Asymmetry imposed by the axial ligands, histidine and methionine, deserves to be considered, as well as the effect of the electric field imposed on the heme by the protein matrix. We recently calculated the electric field at the heme site in cyt *c*, showing that the field is not homogeneous, with the effect of the propionates being especially significant (Köhler et al., 1996; Laberge et al., 1996b).

Solvent Effects on the Spectra. We reasoned that, if the protein existed in two major conformations that are trapped because of an energy barrier, freezing rate and solvent viscosity may affect the splitting. This was not seen; the temperature where resolution for Zn cyt *c* occurred in the two solvents is about the same (Figures 2 and 3), and the values of the splitting in the two solvents are identical. However, the spectral distributions are not the same in the different solvents. Zn cyt *c* in 50% ethylene glycol (Figure 3) shows at least three peaks in the Q_0 region. The lowest-frequency peak can be assigned to the lowest frequency component of the *Q* band; it is the most intense, as is the case with Fe(II) cyt *c* and as expected within the four-orbital model of porphyrin transitions (Shelnutt, 1980). This is not observed in 80% glycerol (Figures 4 and 5) or in the spectra acquired without cryosolvent (Figure 6). For the latter samples, we regard the protein to be acting as an interstitial impurity in the ice crystal, located at the interfaces between crystalline regions of the ice. Because of this, in the absence of the cryosolvent, the protein has a different structure than that it has in liquids or glasses. A change in structure may also occur in 80% glycerol since high concentrations of cryosolvents have been shown to perturb the structure of cytochrome *c* peroxidase (Smulevich et al., 1989). An alternative explanation could be either of the following. (i) The Q_0 spectral envelope could contain two electronic transitions with the e_g degeneracy lifted, in which case the lowest-frequency component Q_x (16 998 cm^{-1}) would have its lower-intensity Q_y complementary band hidden in the next

Q_x band (17 091 cm^{-1}); (ii) the presence of phonon side bands could also add intensity to the 17 091 cm^{-1} band. We are presently investigating this question, as it raises questions about the effect of the matrix on the optical center. Zollfrank et al. (1996) have concluded that the porphyrin in the protein crevice is to a large extent not influenced by the dynamics of the host glass. Also, the decay of the triplet state of zinc porphyrin in the protein was relatively independent of temperature in the range where viscosity changes and where zinc coproporphyrin triplet lifetime is temperature dependent (Figure 11). These results seem to show that the solvent has only a small effect on the porphyrin inside the protein, at least in the triplet state.

Spectral Shifts and Broadening. As the temperature decreases, the absorption of Fe(II) cyt *c* shifts to the blue, as seen in Figure 2. Several possibilities were considered to explain this. The excited state orbital is $e_g(\pi^*)$ and is more delocalized than the ground state orbitals $a_{1u}(\pi)$ and $a_{2u}(\pi)$. For magnesium porphyrin in myoglobin, it was demonstrated that the nuclear positions are displaced in the excited state (Kaposi et al., 1993). Therefore, the excited state molecule would be interacting more with the solvent than would the ground state molecule. As temperature increases, the vibrational levels of the ground state become populated, and the observed transition shift would be expected to shift to the blue. The population of phonon wings would cause spectral broadening, but this in fact predicts the wrong direction of the shift observed in Fe(II) cyt *c*.

A second explanation for the blue shift with decreasing temperature is simply based upon the idea that the protein acts like a glass with an energy landscape described by the two-level system (TLS). As the temperature decreases, the lower-energy levels are populated and the absorptions shift to the blue. This explanation does not necessarily account for the fact that the temperature dependent shift was stronger in Fe(II) cyt *c* than in Zn cyt *c*. The reason may be that there are temperature dependent changes in the iron position such as postulated in myoglobin (Gilch et al., 1995; Sage et al., 1995). In the case of other heme proteins, temperature dependent changes of the heme Soret absorption observed in other heme proteins were interpreted in terms of iron motions (Cordone et al., 1986). A third possibility is that there could be a low-lying level close to the ground state electronic level, which can be thermally populated. A split in the ground state electronic levels was given to explain temperature dependent shifts in the Soret absorption of low-spin ferriheme proteins (Hochstrasser, 1971). But since the Fe(II) cyt *c* ground state term is a singlet, this mechanism to split the electronic level seems unlikely.

Finally, we comment on the widths of the absorption transitions. Since it is well-established that the Zn cyt *c* absorption is inhomogeneously broadened, as supported by both fluorescence line narrowing studies (Koloczek et al., 1987; Logovinsky et al., 1991) and hole burning (Köhler et al., 1996), a comparison of its absorption with that of Fe (II) cyt *c* is instructive. At 10 K, the width of the $Q_{0,0}$ transition is about 95 cm^{-1} for Fe(II) cyt *c* and 93 cm^{-1} for Zn cyt *c* (Table 1). The width of the $Q_{0,0}$ transition for Fe(II) cyt *c* is 95 cm^{-1}, and if homogeneous, this would correspond to a lifetime of 0.18 ps. This value corresponds with the estimated lifetime of Fe(II) cyt *c* of 0.2 ps (Champion & Lange, 1980). Therefore, homogeneous broadening must contribute to the observed widths at the lowest temperatures.

A full analysis of the temperature dependence of cyt *c* absorption would require separation of homogeneous and inhomogeneous effects. The spectral behavior and temperature range being examined will determine which type of protein relaxation process is most influential. The specific heat of proteins is reported to depend linearly on temperature between 40 and 320 K (Mrevlishvili, 1979), implying that a wide variety of protein fluctuations would contribute to the inhomogeneity. In the temperature range of less than 4 K for Zn cyt *c*, Leeson and co-workers found by photon echo experiments that there is more than one motion (Leeson et al., 1994; Leeson & Wiersma, 1995). Hole burning experiments at high resolution showed that structural relaxation in iron-free horseradish peroxidase occurs in a stepwise manner at temperatures as low as 10 K (Zollfrank et al., 1991a). These kinds of motions would lead to the spectral broadness that is seen below 100 K and contribute to the overall broadness. At higher temperatures, other kinds of motions would play a role. For example, the phenylalanine flip occurs at temperatures around 0 °C (Burns & La Mar, 1981). The heme has more than 50 contacts with the protein, and a change in atomic position of any of these will produce a shift in the absorption spectra. If there are three positions for each atomic group, as was seen for CO bound to myoglobin, there will be 3 exp 50 possible "subconformations", an astronomical number. However, many of the motions are essentially oscillatory; in this case, there would be a continuous distribution. For both cases, the observation of essentially a continuum in the distribution of transition energies can be attributed to the motion of the atoms around the porphyrin. Fluctuations of the atomic positions in a protein have large effects on electrostatics (Wendoloski & Matthew, 1989; Northrup et al., 1990; Langsetmo et al., 1991; Köhler et al., 1996; Laberge et al., 1996a). Changes in the field will change the splitting and will manifest themselves in inhomogeneous broadening of the absorption spectrum of the protein-bound chromophore. In fact, that the JT splitting shows a distribution of values in Zn cyt *c* is evident from the temperature dependence of the EPR spectrum of the triplet excited state which indicated that there are a range of δ_{JT} values (Angiolillo & Vanderkooi, 1995).

In summary, the model that emerges from these spectral studies and previous work is that cyt *c* exists with essentially a continuous distribution of subconformations. These are not due to any large change in the protein conformation but arise from the fluctuations of the side chain residues and the peptide chain. The fluctuations of the surrounding atoms contribute to the inhomogeneous broadening and the widths of the transitions; in that sense, the protein is liquid-like. However, the protein environment closely resembles a crystal in terms of a defined crystal field imposed. The electric field of the protein reduces the heme symmetry, and the data seem consistent with the view that the spectral splitting arises from the distortion that the polypeptide chain imposes upon the metalloporphyrin, causing *xy* asymmetry. This effect is rather large in the protein, and since the protein is undergoing fluctuations, the magnitude of the split itself fluctuates, leading to the broadness of the transition seen at the lowest temperature. Finally, it is commonly known that the $Q_{0,0}$ bands of many Fe(II) heme proteins show splitting even at room temperature. This suggests that splitting can be used

to indicate asymmetry in the heme chromophoric environment.

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