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Crystal Spectra of a Heme and Some Heme–Protein Complexes*

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ABSTRACT: The crystal spectra of mesoporphyrin IX iron(III) methoxide, ten myoglobin derivatives, and cytochrome *c* peroxidase have been obtained. The comparison between these spectra and the corresponding solution spectra show that in all cases there is a reduction in intensity of absorption bands in the solid state but that in most cases the energy of the spectrum is virtually unchanged. Myoglobin fluoride and cytochrome *c* peroxidase are exceptions. It is proposed that the crystallization of a heme protein always causes a shift in the equilibrium position, high-spin form $\rightleftharpoons$ low-spin form, toward low-spin forms but that changes in conformation can also be involved. The use of polarized single-crystal spectra has permitted an assignment of all the absorption bands in the spectra. The transitions are all polarized in the heme plane, showing them to have the same character as the $\pi-\pi^*$ transitions.

The crystal spectra of both small molecules and proteins are of interest in two very different fields of study. First an unpolarized crystal spectrum can be compared directly with a solution spectrum. Any changes which are then observed can be interpreted in terms of conformation changes, restrictions of vibrations, or cooperative effects. Details of one such study on a small model compound, $PtCl_4^{2-}$, are described by Day *et al.* (1965). The application of this comparison to protein molecules is particularly desirable for at present it is assumed that X-ray crystal studies in the solid state are directly relevant to solution studies despite the fact that this has not been put to careful experimental test. In this paper we shall examine the crystal spectra of several myoglobin derivatives of known crystal structure in an effort to establish whether or not distinct differences between solid and solution phases need to be postulated. We have also examined the spectra of cytochrome *c* peroxidase in both phases although the crystal structure of this protein is not known. In both proteins the established sensitivity of the spectra of high-spin iron(III) porphyrin compounds to changes in the environment of the iron atom make the systems ideal for our studies. Furthermore, it is already known that there are differences in reactivity between solid and solution phases of myoglobin compounds (Chance *et al.*, 1966).

The second field of interest concerns the theory of the absorption spectra of metal porphyrins. Although the *visible* spectra of porphyrins have been extensively studied and subjected to theoretical treatment (Braterman *et al.*, 1964; Day *et al.*, 1964; Gouterman, 1961; Weiss *et al.*, 1965), the near-infrared region has been neglected. The origin of the weak band observed at about 10,000 cm^{-1} in high-spin iron(III) porphyrins has not been conclusively established, and $\pi-\pi^*$ triplets have not been observed in absorption. Polarized single-crystal spectra are of particular value in making such assignments as we shall see below.

Materials

The mesoporphyrin IX iron(III) methoxide (dimethyl ester) was kindly supplied by Dr. W. S. Caughey. Myoglobin crystals were a gift from Dr. C. Nobbs. The myoglobin derivatives were obtained by bathing crystals of the acid metmyoglobin in solutions of different simple salts. The concentrations of the solutions were chosen so as to give at least 99.0% formation of the required derivative as estimated from solution equilibrium data. The treatment of the crystals lasted at least 24 hr and in the case of the thiocyanate it was shown that bathing for longer periods did not alter the crystal spectrum. Each compound was measured at least twice. The cytochrome *c* peroxidase crystals were a gift from Professor T. Yonetani.

The single-crystal polarized spectra were obtained using an apparatus which is essentially a microspectrophotometer employing a reflection microscope and a polarizer. With it crystals between 0.2 and 1.0 mm in length can be studied. The instrument will be described in detail later.

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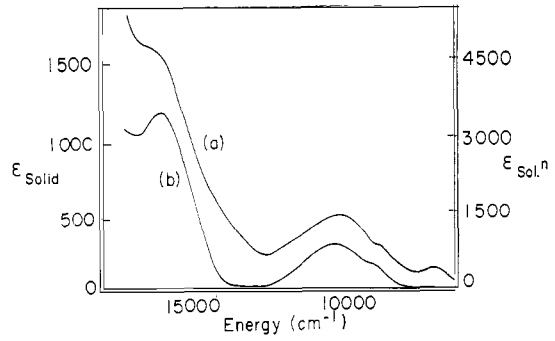

FIGURE 1: (a) Unpolarized crystal spectrum of metmyoglobin and (b) solution spectrum. ϵ is in liters per reciprocal moles per reciprocal centimeters. Note change of scale on ordinate.

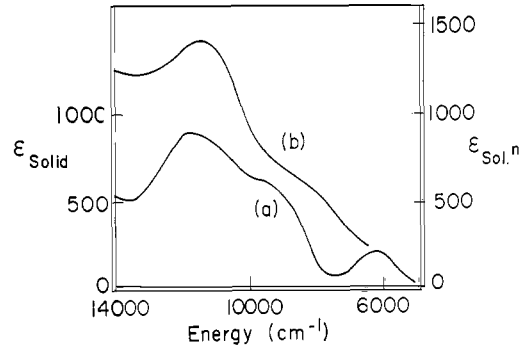

FIGURE 2: Comparison between single-crystal spectrum of metmyoglobin fluoride (a) and its solution spectrum (b).

Crystal and Solution Spectra

Results

Figure 1 shows the unpolarized crystal spectrum and the solution spectrum of acid metmyoglobin, showing the first visible band as well as the near-infrared absorption. The intensities of both bands have fallen considerably compared with the solution spectrum, and the first visible band, which appears as a distinct peak at 15,800 cm^{-1} in solution, has degenerated into a shoulder. Table I compares the positions and intensities of bands in crystal spectra with those in solution spectra for nine further myoglobin derivatives. The intensities in the crystal spectra are appreciably lower than in solution, except for the first visible band of the azide.

TABLE I: Crystal Spectra of Myoglobins (solution data[a] in parentheses).

Compound	λ_{max} (cm^{-1})	ϵ_{max} (l. $mole^{-1}$ cm^{-1})
Met (H_2O)	10,000 (10,000)	520 (~860)
	16,000	1,500 (3,900)
Hydroxide	12,200 (12,200)	450 (~700)
Fluoride	11,600 (11,600)	1,000 (1,400)
Cyanate	10,500 (10,500)	730 (1,010)
	16,000	2,250 (4,750)
Thiocyanate	10,000 (10,000)	575 (~830)
	16,000	2,900 (3,700)
Nitrite	10,500 (10,500)	500 (~800)
	16,000	1,600 (4,000)
Azide	10,000 (10,000)	250 (~350)
	16,000	430 (300)
Acetate	9,800 (10,000)	490 (~830)
	16,000	1,500 (3,800)
Formate	10,600 (10,600)	785 (1,050)
	16,000	2,000 (5,100)

[a] Solution intensities are those given by Davies (1963).

However, the positions of the near-infrared bands are unchanged in the crystal with the exception of the case of fluoride (Figure 2). Considerable increase in resolution is seen in all the crystal spectra. The exact position of the first visible band is difficult to locate with precision in the crystal spectra.

The single-crystal spectrum and solution spectrum of cytochrome *c* peroxidase is shown in Figure 3. We were unable to obtain accurate extinction coefficients but the first visible band again appeared as a shoulder. The near-infrared absorption was resolved into three components at 11,500, 10,600, and 9500 cm^{-1}, which are not seen in solution. The intensity of the bands in the 10,000- and the 16,000-cm^{-1} regions of the spectrum are markedly lower in the solid state. The Soret region of the spectrum also undergoes considerable change on crystallization and even the spectra of frozen solutions of this enzyme[1] show that cooling a solution produces a similar considerable effect on the spectrum as crystallization. In the electron spin resonance (esr) spectrum of cytochrome *c* peroxidase (Yonetani *et al.*, 1966a,b) there is a change in the relative intensities of absorption in different regions of the spectrum going from a microcrystalline sample to a solution at the same temperature.

Discussion

Both the infrared and the first band in the visible spectrum are characteristic of high-spin ferric porphyrins and their intensities can be used as a rough guide to the amounts of the high-spin states present in equilibrium with the low-spin forms. In solution ferric myoglobins are considered to be mainly high spin, with the exception of the azide which contains a large proportion of the low-spin form at room temperature. Our results indicate that in all the myoglobin compounds the proportion of high-spin Fe(III) is lower in the crystal than in solution. Since the center of the near-infrared band, which is quite sensitive to the axial ligand, re-

[1] T. Yonetani, private communication.

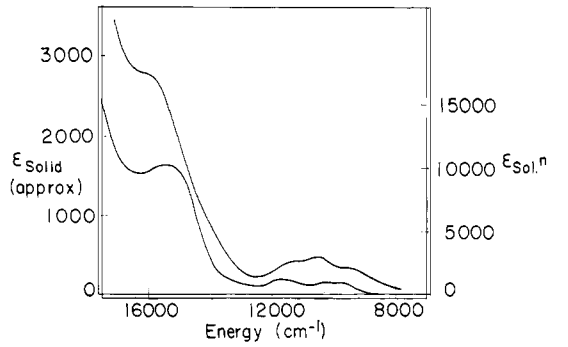

FIGURE 3: Single-crystal spectrum of cytochrome *c* peroxidase (upper curve) and its solution spectrum (lower curve). Note change of scale on ordinate.

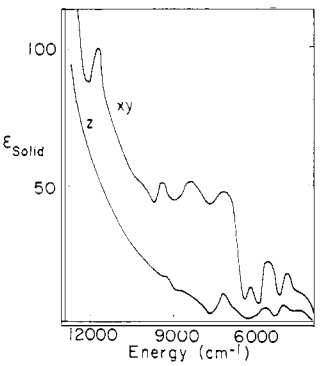

FIGURE 4: Polarized crystal spectrum of mesoporphyrin IX (dimethyl ester) iron(III) methoxide.

mains virtually unchanged in all but the case of the fluoride, where but small changes are seen, it appears that no gross differences exist between the environment of the high-spin metal ion in solution and in the crystal. The change in the balance between the percentage of the two spin states can probably be accounted for by a small conformational change, since the balance between the high- and low-spin forms is evidently most delicate. There is definitely some change in the ground state between solution and crystal no matter what additional changes occur in transition states (Chance *et al.*, 1966) and this change may be sufficient to account for kinetic differences.

These results have some general implications for the discussion of the reactivity of proteins on the basis of their crystal structures. While it is undoubtedly true that the changes in going from myoglobin crystals to solutions are small as seen in the spectra, they are always discernable and, whatever causes these changes, it does bring about marked changes in reactivity. The changes in spectra of cytochrome *c* peroxidase are considerably larger but the effect on reactivity of phase change is not yet known. Unless a determined attempt is made to prove that other proteins have the same geometry in solution and in the crystalline state by one method or another it does not seem to be an essentially safe conclusion to assume that no such differences exist.

Polarized Spectra

Results

In mesoporphyrin IX iron(III) methoxide (dimethyl ester), the crystal structure of which has been determined (Hoard *et al.*, 1965), the near-infrared absorption was found to be polarized in the same direction as that in the visible region (Figure 4).

The crystal habit and structure of sperm whale myoglobin (Kendrew and Parrish, 1957) make it particularly suitable for polarization studies. The high molecular weight enables relatively long path lengths to be used since the effective concentration of the chromophore is reduced. Figure 5 shows the polarized crystal spectrum of acid metmyoglobin in the near-infrared spectrum. The band at 10,000 cm^{-1} is clearly polarized in the plane of the porphyrin ring since the greater absorption occurs when the electric vector is parallel to the crystallographic *b* axis. The excited state must again be of the same symmetry as π^* and charge-transfer states which account for the visible absorption. The shoulder at 8700 cm^{-1} is observed in most of the myoglobin derivatives we have studied, and the near-infrared absorption would appear to have at least two, possibly three, components. The weak unpolarized band at 6500 cm^{-1} is assigned to a water overtone. In addition to metmyoglobin, we have examined the azide, fluoride, hydroxide, thiocyanate, cyanate, nitrite, formate, and acetate, all prepared by the diffusion method, and in each case the in-plane polarization of the near-infrared band was confirmed.

Discussion

The polarization of all the bands in the heme spectra from 25,000 to 5000 cm^{-1} is now known to be in plane. The proposed character of the transitions at 20,000, 16,000, and 10,000 cm^{-1} as being mixed charge-transfer and ligand bands (Day *et al.*, 1964) is supported by these polarization data for the symmetry of all the transitions were of necessity the same and, as the

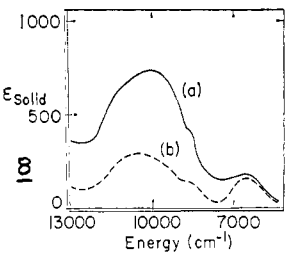

FIGURE 5: Crystal spectrum of metmyoglobin with electric vector parallel (a) or perpendicular (b) to *b* axis. ϵ is in liters per reciprocal moles per reciprocal centimeters.

transitions of the ligand are in plane, were required to be in-plane. In addition to these bands there are a series of very weak bands ($\epsilon > 50$) at 7200, 8300, and 9400 cm^{-1} in the spectra of the mesoporphyrin IX iron(III) methoxide crystals. These are possibly spin-forbidden components of the lowest electronic excited state, and all are polarized in the same direction, *i.e.*, in the heme plane. Sharp weak bands at lower wavenumbers are assigned to vibrational overtones.

Now that it is known that all the transitions of Fe(III)–heme complexes are in plane the discovery of out-of-plane transitions associated with Fe(III)–heme complexes in proteins implies electron excitation between the heme and other groups of the protein. Thus it is of great importance to establish the polarization of the 695-mμ band of cytochrome *c*. If it is confirmed (Eaton and Hochstrasser, 1966) that this band is polarized perpendicular to the heme plane, then the absorption is definitely not due to a transition of the same kind as we have observed in this paper. It could well be a charge-transfer band, protein group, *i.e.*, tyrosine or tryptophan, to iron(III) porphyrin. We intend to extend our measurements on single crystals to liquid-hydrogen temperature where the components of all the bands will be better resolved.

Acknowledgment

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CORRECTIONS

In the paper "Faster Oxidation of Tyrosine-26 of Oxidized B Chain of Insulin by Tyrosinase," by Joseph G. Cory and Earl Frieden, Volume 6, January 1967, an error appears in the equation on p 119. The equation should read

$$\frac{(Tyr_1)_t + (Tyr_2)_t}{(Tyr_1)_0 + (Tyr_2)_0} = \frac{(Tyr_1)_0}{(Tyr_1)_0 + (Tyr_2)_0} e^{-2.6(10^{-2})t} + \frac{(Tyr_2)_0}{(Tyr_1)_0 + (Tyr_2)_0} e^{-2.0(10^{-3})}$$

instead of

$$\frac{(Tyr_1)_t + (Tyr_2)_t}{(Tyr_1)_0 + (Tyr_2)_0} = \frac{(Tyr_1)_t}{(Tyr_1)_0 + (Tyr_2)_0} e^{-2.6(10^{-2})t} + \frac{(Tyr_2)_t}{(Tyr_1)_0 + (Tyr_2)_0} e^{-2.0(10^{-3})}$$

In the paper "Ultraviolet Irradiation Effects in Poly-L-tyrosine and Model Compounds. Identification of Bityrosine as a Photoproduct," by S. S. Lehrer and G. D. Fasman, Volume 6, March 1967, the legend for Figure 1 on p 759 has been transposed. It should read: (a) Normalized absorption spectrum after irradiation; (a′) absorption before irradiation relative to (a).