may contain additional peptide chains terminated by an acylamino group or by a residue of pyrrolidonecarboxylic acid. These would have to be bound covalently to the chains with free amino termini by disulfide bridges or very firmly by noncovalent interactions. We consider these possibilities unlikely.

The catalytic subunit of molecular weight 1.0×10^5 appears to be a dimer of identical chains, each with molecular weight 5.0×10^4, a single amino-terminal alanine and a single binding site for succinate. In this regard, it is pertinent to note that the catalytic subunit dissociates into smaller units in denaturing solvents (Changeux *et al.*, 1967).

Acknowledgment

We are most grateful to Dr. John Gerhart for providing stocks of the derepressed mutant of *E. coli* and for allowing us to use the purification procedure before publication. Dr. Charles Yanofsky very kindly allowed us to use his equipment for electrophoresis and chromatography and gave valuable advice in the techniques of mapping peptides.

References

Acher, R., and Crocker, C. (1952), *Biochim. Biophys. Acta 9*, 704.

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 831.

Changeux, J.-P., and Gerhart, J. C. (1968), *Biochemistry* (in press).

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1967), *in* Regulation of Nucleic Acid and Protein Biosynthesis, Koningsberger, V. V., and Bosch, L., Ed., New York, N. Y., Elsevier, p 344.

Fruchter, R. G., and Crestfield, A. M. (1965), *J. Biol. Chem. 240*, 3875.

Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2886.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Helinski, D. R., and Yanofsky, C. (1962), *Biochim. Biophys. Acta 63*, 10.

Hunninghake, D., and Grisolia, S. (1966), *Anal. Biochem. 16*, 200.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Rodbell, M., and Fredrickson, D. S. (1959), *J. Biol. Chem. 234*, 562.

Stark, G. R. (1967), *Methods Enzymol. 11*, 125.

Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.

Weigert, M. G., and Garen, A. (1965), *J. Mol. Biol. 12*, 448.

Crystal Spectra of Some Ferric Hemoproteins*

P. Day, D. W. Smith, and R. J. P. Williams

ABSTRACT: Polarized crystal spectra of the low-spin compounds ferricytochrome *c* and ferrimyoglobin cyanide are reported. In both spectra a weak band at approximately 8000 cm^{-1} was found to be polarized in the heme plane. In cytochrome *c* there is a further band at 10,700 cm^{-1} which indicates that the heme complex contains a few per cent of the high-spin form. Measurement of the spectra of metmyoglobin and ferricytochrome *c* crystals which had been bathed in D_2O confirm our previous assignment of the 6500 cm^{-1} band as a water overtone. The relationship between the band intensities in the crystal and solution is discussed. The lower intensity of the near-infrared absorption in crystalline myoglobin derivatives compared with that found in solution spectra may result from splitting of the degenerate excited state.

It is generally considered that the absorption spectra of heme complexes can be used as a guide to the spin state of the central metal atom (Williams, 1956). In a previous paper (Day *et al.*, 1967) we used this argument in a comparison between the solution and crystal spectra of a series of myoglobin derivatives in high- or mixed-spin states. We have now extended our measurements to myoglobin cyanide and ferricytochrome *c*, which are believed to be of the low-spin type from magnetic susceptibility measurements. Single crystals of the latter compound have already been studied from 28,000 to 13,000 cm^{-1} by Eaton and Hochstrasser (1967), but no measurements were reported in the near-infrared region. Crystal spectra in

* From the Inorganic Chemistry Laboratory, South Parks Road, Oxford, England. *Received September 12, 1967.* D. W. S. thanks the Science Research Council (U. K.) for a research studentship. The work was supported by the Medical Research Council.

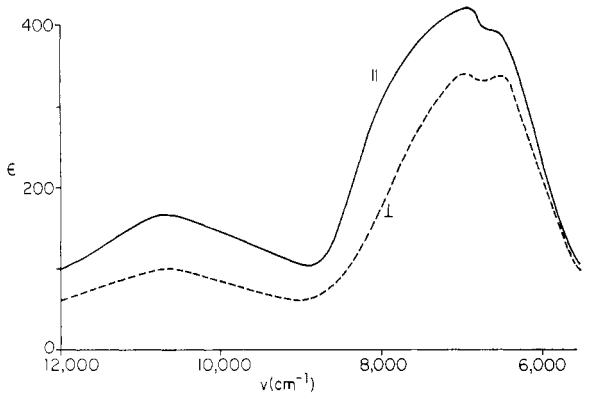

FIGURE 1: Polarized crystal spectrum of ferricytochrome *c*, with electric vector parallel (||) and perpendicular (⊥) to the *c* axis. *E* is in 1 mole^{-1} cm^{-1}.

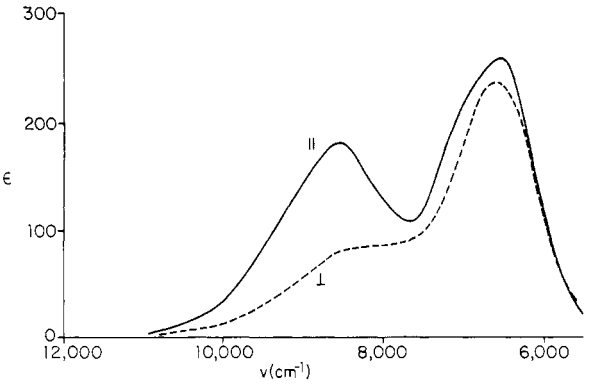

FIGURE 3: Polarized crystal spectrum of deuterated metmyoglobin, with electric vector parallel (||) and perpendicular (⊥) to the *b* axis. *E* is in 1 mole^{-1} cm^{-1}.

the latter region give more information about spin states. Hemoprotein crystals also have an absorption band around 6500 cm^{-1} which we have assigned to a water overtone, and we considered it informative to examine this band after replacing water in the crystal by D_2O. We also present a more detailed discussion of our basis for comparing extinction coefficients in crystals and solutions and suggest an alternative explanation for the lower intensities found in crystals to the one we gave previously.

Materials and Methods

Crystalline horse heart cytochrome *c* was generously given by Dr. E. Margoliash. Ferrimyoglobin cyanide was prepared by bathing metmyoglobin crystals in a 10:1 excess of aqueous sodium cyanide. Deuterated metmyoglobin and ferricytochrome *c* crystals were prepared by replacing the mother liquor with a saturated solution of ammonium sulfate in D_2O, and allowing the crystals to stand for several days.

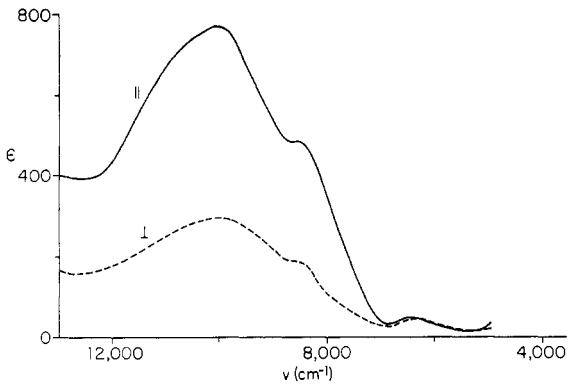

FIGURE 2: Polarized crystal spectrum of ferrimyoglobin cyanide, with electric vector parallel (||) and perpendicular (⊥) to the *b* axis. *E* is in 1 mole^{-1} cm^{-1}.

Spectra were measured at room temperature by means of a microspectrophotometer constructed in this laboratory (Barnes and Thomson, 1967). Detailed procedures were as previously described.

Results

The crystal spectrum of ferricytochrome *c* was measured with the electric vector parallel and perpendicular to the *c* axis. Extinction coefficients were calculated from the molar concentration of 0.046 M, obtained from unit cell data for B-type crystals (Margoliash and Schejter, 1966), and the measured path length. The spectrum is shown in Figure 1. The *c* direction is seen to be that of greater absorption, and the dichroic ratio is similar to that found in the region 16,000–28,000 cm^{-1} (Eaton and Hochstrasser, 1967), where the bands are considered to be polarized in the heme plane. Thus the bands at 10,700 and 7000 cm^{-1} are also in-plane polarized.

The crystal spectrum of ferrimyoglobin cyanide was measured with the electric vector parallel and perpendicular to the *b* axis, with the light incident on the (001) face. The spectrum is shown in Figure 2. Extinction coefficients were calculated from the molar concentration of 0.049 M, obtained from unit cell data for A-type crystals (Kendrew and Parrish, 1956) and the measured thickness. The heme-group orientation for myoglobin as given by Bennett *et al.* (1957) shows that the *b* direction is that of greater absorption for transitions polarized in the heme plane, so that the band at 8500 cm^{-1} is in-plane polarized while the band at 6500 cm^{-1} is nondichroic.

Polarized crystal spectra of deuterated metmyoglobin and ferricytochrome *c* were obtained in the same way and are shown in Figures 3 and 4, respectively. Absorption in the region of 6500 cm^{-1} is appreciably reduced by replacing water by D_2O. Otherwise the spectra are not significantly affected, except that the low-energy band in ferricytochrome *c* now appears at 7300 cm^{-1}.

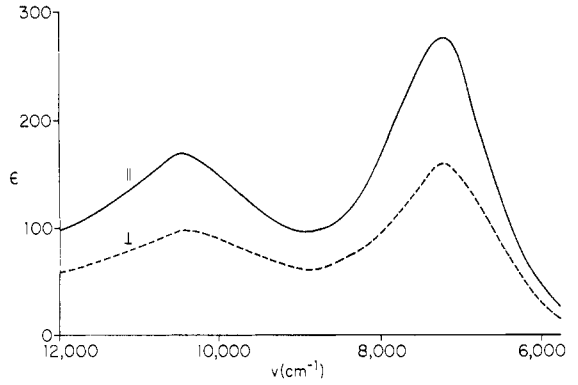

FIGURE 4: Polarized crystal spectrum of deuterated ferricytochrome *c*, with electric vector parallel (∥) and perpendicular (⊥) to the *c* axis. *E* is in 1 mole^{-1} cm^{-1}.

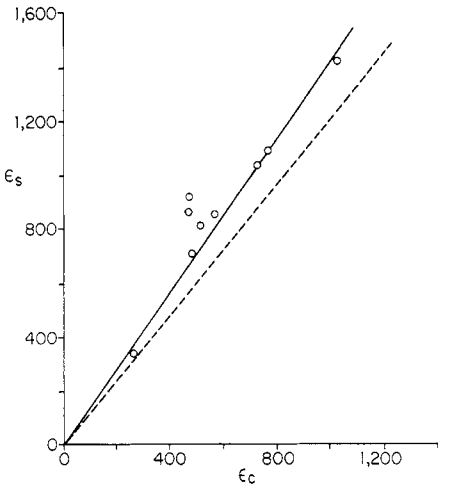

FIGURE 5: Extinction coefficient of the near-infrared bands of metmyoglobin derivatives in solution (E_s) and in the crystalline state (E_c).

Discussion

Protein crystals usually contain about 50% water, and we have previously assigned the band at 6500 cm^{-1} to a water overtone involving the OH stretching frequency. The near-infrared spectrum of liquid water (Curcio and Petty, 1951) suggests that a band of this energy and intensity would be expected in protein crystals, assuming that the structure of water in the crystal is similar to that in the pure liquid. The spectroscopic consequences of deuteration are in agreement with this assignment, since the molar extinction coefficient at 6500 cm^{-1} is reduced from 170 to 50 in metmyoglobin, and the shoulder at 6500 cm^{-1} in ferricytochrome *c* disappears. However, the shoulder at 8700 cm^{-1} in metmyoglobin is unaffected, and thus cannot be attributed to water. Figure 4 gives a more accurate spectrum of ferricytochrome *c* than Figure 1 where the water absorption interferes. The dichroic ratio is uniformly 1.7, similar to the value obtained in the visible region by Eaton and Hochstrasser (1967).

Weak bands in the region of 6000–7000 cm^{-1} have been reported in aqueous solution for a number of myoglobin derivatives (Hanania *et al.*, 1966) but these also probably arise from water absorption, since the sample and reference cells cannot be matched so perfectly as to cancel out the very strong solvent absorption completely.

After allowance is made for the water absorption, the positions of the near-infrared bands in both ferricytochrome *c* and ferrimyoglobin cyanide are in fair agreement with those found in solution in the region 9000–7000 cm^{-1} by Hanania *et al.* (1966) and Davies (1963). The magnetic susceptibility of ferrimyoglobin cyanide (Beetlestone and George, 1964) indicates that this is a low-spin compound, and the same is true for ferricytochrome *c*, except at very low pH (Theorell, 1941). Thus the substantial change in electronic structure which occurs on change of spin state is reflected in the near-infrared spectrum as well as in the visible region (Williams, 1956). The in-plane polarized bands at 8500 cm^{-1} in ferrimyoglobin cyanide and at 7300 cm^{-1} in ferricytochrome *c* are probably the spin-forbidden analogs of the lowest energy bands in the visible region. The weak in-plane polarized band at 10,700 cm^{-1} in ferricytochrome *c* would appear to correspond to the band found in this region in high-spin ferric hemoproteins, indicating the presence of a few per cent of the high-spin form. Thus ferricytochrome *c* appears to exist in a balance of spin states similar to that recently observed by electron paramagnetic resonance measurements in cytochrome b_2 (Ehrenberg and Bois-Poltoratsky, 1967).

The intensities of the bands in the region 7000–9000 cm^{-1} are comparable to those found in solution by Hanania *et al.* (1966) and Davies (1963). Close comparison of crystal and solution intensities is not possible in these cases since the low extinction coefficients are not easily measured with precision. We cannot say whether or not there are significant changes in intensity on crystallization, such as we found in a number of high-spin and mixed-spin compounds (Day *et al.*, 1967). It was there suggested that these differences between crystal and solution spectra were related either to slight changes in the position of equilibrium between the high-spin and low-spin forms or to small conformational changes. We now give a more rigorous treatment of the relationship between band intensities in crystals and solutions.

Band Intensities in Myoglobin Crystals

At a specified wavelength in an isolated molecule, we can define the extinction coefficients E_x, E_y, and E_z, where the subscripts refer to the direction of the electric vector. In solution, where all molecular orientations are presented to the incident electric vector, we observe the isotropic extinction coefficient $E_i = {}^1/_3(E_x + E_y + E_z)$. If the chromophore is taken to be of D_{4h} symmetry, then $E_x = E_y = E_{xy}$. For absorption polar-

ized in the molecular plane, $E_z = 0$. Then $E_i = {}^2/_3 E_{xy}$.

For a monoclinic crystal with orthogonal crystal axes a, b, and c^*, the corresponding extinction coefficients E_a, E_b and E_{c^*} are related to E_x, E_y, and E_z by $E_a + E_b + E_{c^*} = E_x + E_y + E_z$ (Albrecht and Simpson, 1955). Now consider an A-type myoglobin crystal flattened on the 001 face. If the incident electric vector makes an angle ϕ with the b axis, then

$$E = E_b \cos^2 \phi + E_a \sin^2 \phi$$

A vector normal to the heme plane makes angles of 28, 69, and 73° with the a, b, and c^* axes, respectively (Bennett *et al.*, 1957). Thus $E_b = \sin^2 69° E_{xy}$ and $E_a = \sin^2 28° E_{xy}$. If the light incident on the 001 face is unpolarized, $E = E_{av}$ is given by

$$E_{av} = \int_0^\pi E d\phi \Big/ \int_0^\pi d\phi = {}^1/_2(E_a + E_b) = 0.55 E_{xy}$$

Since $E_i = {}^2/_3 E_{xy}$, we expect a reduction in the extinction coefficient by a factor of 0.83 in the crystal if oscillator strengths remain the same. For the near-infrared band around 10,000 cm^{-1} in high-spin myoglobin derivatives the reduction factors are found to lie between 0.6 and 0.75. The ratios of crystal extinction coefficients to those in solution are lower still for the visible band around 16,000 cm^{-1}, but there is a possibility of some flattening of this band by stray light. In Figure 5 solution extinction coefficients (E_s) for the near-infrared band around 10,000 cm^{-1} in a number of myoglobin derivatives are plotted against the corresponding crystal intensities (E_c). The data are taken from our previous discussion of myoglobin crystal spectra (Day *et al.*, 1967), and the broken line has a slope of 0.83, in accordance with the above theoretical treatment. The deviations of the experimental points from this line are significant, and cannot readily be accounted for by experimental error. The applicability of Beer's law was confirmed for myoglobin nitrite, of which a wide range of crystal thicknesses was available.

In a discussion of the polarized crystal spectrum of cytochrome c in the visible and near-ultraviolet regions (Eaton and Hochstrasser, 1967), a variation in the dichroic ratio in different regions of the spectrum was noted. These authors pointed out that this could be explained by a lowering in symmetry leading to a splitting of the degeneracy of the E_u excited state. In this circumstance the observed bands would no longer be single xy-polarized transitions but the result of superimposing closely spaced separately x- and y-polarized components, not necessarily of the same intensity. Such a splitting in myoglobin would render our above theoretical treatment of crystal intensities invalid, since we have assumed that $E_x = E_y = E_{xy}$.

A possible source of splitting of the excited state would be the participation of the axial imidazole ligand in π bonding to the metal. The effective symmetry of the chromophore would then be reduced to D_2, splitting the E_u excited state to b_2 and b_3.

Even in the ground state the possibility of nonequivalent x and y axes cannot be excluded as the broad electron spin resonance signal at $g \sim 6.0$ is asymmetric (Ehrenberg, 1962). Thus we cannot say that the observed differences in crystal and solution spectra are due to differences in the physical environment of the heme group or are due to removal of degeneracy in the ground or excited state of the molecule present in both phases.

Unless the differences between solution and crystal spectra are larger than those seen in the myoglobin series of compounds real difficulty is bound to be experienced in interpretation using absorption spectra. Cytochrome c peroxidase is a clear-cut case where differences exist (Day *et al.*, 1967).

References

Albrecht, A. C., and Simpson, W. T. (1955), *J. Chem. Phys. 23*, 1480.

Barnes, J. C., and Thomson, A. J. (1967), *J. Sci. Instr.* (in press).

Beetlestone, J., and George, P. (1964), *Biochemistry 3*, 707.

Bennett, J. E., Gibson, J. F., and Ingram, D. J. E. (1957), *Proc. Roy. Soc. (London) A240*, 67.

Curcio, J. A., and Petty, C. C. (1951), *J. Opt. Soc. Am. 41*, 302.

Davies, R. C. (1963), Ph.D. Thesis, Oxford University, England.

Day, P., Smith, D. W., and Williams, R. J. P. (1967), *Biochemistry 6*, 1563.

Eaton, W. A., and Hochstrasser, R. M. (1967), *J. Chem. Phys. 46*, 2533.

Ehrenberg, A. (1962), *Arkiv Kemi 19*, 119.

Ehrenberg, A., and Bois-Poltoratsky, R. (1967), *in* Symposium on Cytochromes, Okunuki, K., and Kamen, M. D., Ed., Osaka, Japan, Tokyo University, p 309.

Hanania, G. I. H., Yeghiayan, A., and Cameron, B. F. (1966), *Biochem. J. 98*, 189.

Kendrew, J. C., and Parrish, R. G. (1956), *Proc. Roy. Soc. (London) A238*, 305.

Margoliash, E., and Schejter, A. (1966), *Advan. Protein Chem. 21*, 113.

Theorell, H. (1941), *J. Am. Chem. Soc. 63*, 1820.

Williams, R. J. P. (1956), *Chem. Rev. 56*, 299.