Analysis of the Visible Spectra of some Sperm-Whale Ferrimyoglobin Derivatives

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(Received 22 July 1968)

The visible spectra of sperm-whale metmyoglobin and its fluoride, hydroxide, cyanide, azide, cyanate, formate, acetate, thiocyanate and nitrite derivatives were measured and resolved into Gaussian components. A linear correlation between the intensity of the band at 18500 cm^{-1} and the magnetic susceptibility is found, suggesting that the band is the β -band of the low-spin form. The analysed intensity of this band can be used to determine the relative proportions of the two spin states present. The spectra of the pure high-spin and low-spin components of each derivative can be reconstructed. Correlations between the energy of the near-infrared band and the visible bands of the high-spin form, and between the position and intensity of the 16000 cm^{-1} band, are demonstrated. The relationship between the position of the Soret band and the magnetic susceptibility can be explained in terms of the equilibrium between spin states.

The absorption spectra of haems and haemoproteins, and the electronic structure of the haem chromophore, have been the subjects of much discussion (Brateman, Davies & Williams, 1964; Zerner, Gouterman & Kobayashi, 1966). The spectra of ferrous haems and haemoproteins are now reasonably well understood, but the ferric compounds are much more complicated for two reasons. In the first place, new bands, not encountered in other metalloporphyrins, appear in the spectrum. These have been assigned to ligand-to-metal charge-transfer transitions, subject to configuration interaction with the intra-ligand $\pi-\pi^*$ transitions (Day, Scrogg & Williams, 1964). The second difficulty arises from the existence of an equilibrium between the high-spin and low-spin forms, each with distinct spectra. George, Beetlestone & Griffith (1961) correlated the spectra and paramagnetic susceptibilities of some ferric haemoprotein hydroxides. However, to assign the visible bands of a wider range of derivatives, it is clearly necessary to separate the contributions of the two spin states to the spectrum. Following the procedure of Drabkin (1961), who showed that the spectra of many haemoproteins could be resolved into Gaussian components, we present an analysis of the visible spectra of some ferrimyoglobin derivatives.

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MATERIALS AND METHODS

Sperm-whale metmyoglobin, crystallized from saturated $(\text{NH}_4)_2\text{SO}_4$ solution, was kindly supplied by Dr C. Nobbs. An approximately 1 mM stock solution, pH 6.5, was prepared and standardized spectrophotometrically from the data of Hanania, Yeghiayan & Cameron (1966). Derivatives, in which the water molecule co-ordinated to the metal was replaced by an anion, were prepared by adding the appropriate sodium or potassium salt to the stock solution until the spectrum was unchanged by further addition of solid.

Spectra were determined at room temperature on a Unicam SP.800 spectrophotometer in the region $14000-20000\text{ cm}^{-1}$. The curves obtained were analysed into Gaussian components by the use of a programme written in Algol for the Oxford University KDF 9 computer by Mr B. D. Bird of this Laboratory. For the cyanide 12 points, chosen between 15000 and 19000 cm^{-1} , were fitted to two Gaussian curves, and for the azide 20 points, between 15000 and 19000 cm^{-1} , were fitted to three Gaussian curves. All other spectra were fitted to four curves and at least 25 points, between 14000 and 20000 cm^{-1} , were taken in each case. The computer performed a least-squares fitting procedure, which was continued until further cycles produced a negligible change in the parameters.

RESULTS

The positions and intensities of the bands found to fit the spectra satisfactorily are given in Table 1. The root mean square deviation between observed and calculated millimolar extinction coefficients,

Table 1. *Analysed ferrimyoglobin spectra*

Axial ligand	ν (cm. ⁻¹)	ϵ_{mM}	ν (cm. ⁻¹)	ϵ_{mM}	ν (cm. ⁻¹)	ϵ_{mM}	ν (cm. ⁻¹)	ϵ_{mM}
H ₂ O	15690	3.00	16840	2.20	18300	1.19	19940	9.62
F ⁻	16590	6.54	17110	0.70	18400	0.29	20310	9.3
H·CO ₂ ⁻	15790	3.52	16920	3.02	18320	1.47	19960	9.55
CH ₃ ·CO ₂ ⁻	15640	2.54	16640	2.51	18110	1.61	19890	9.49
NO ₂ ⁻	15820	3.46	17060	3.14	18380	2.50	20230	7.60
SCN ⁻	15650	3.18	16860	2.77	18360	1.90	19790	9.07
OCN ⁻	15780	4.16	16990	3.75	18470	1.09	19850	8.53
OH ⁻	16780	4.16	17240	3.17	18520	3.66	20660	8.01
N ₃ ⁻	15700	0.85	17200	4.34	18510	8.70	—	—
CN ⁻	—	—	17160	2.07	18540	10.35	—	—

ϵ_{mM} , was between 0.03 and 0.06 mm⁻¹ cm.⁻¹, little more than the experimental error in the observed intensities.

DISCUSSION

Bands near 17000 and 18500 cm.⁻¹ are present in all the ferrimyoglobin derivatives studied, but these are most intense in those compounds that are believed to be largely low-spin in character, i.e. the azide and cyanide. The bands at 16000 and 20000 cm.⁻¹ appear to be characteristic of the high-spin form (Brill & Williams, 1961). Spectroscopic studies of other metalloporphyrins have shown that the β -band of a porphyrin is little affected in either position or intensity by substitution at the metal atom, whereas the intensity of the α -band is strongly dependent on the identity of the axial ligand (Brateman *et al.* 1964). Thus, if we adopt the view that the bands seen at 17000 and 18500 cm.⁻¹ are to be identified with the α - and β -bands of the low-spin form, we would expect to find a linear relationship between the intensity of the 18500 cm.⁻¹ band and the paramagnetic susceptibility for a series of axially substituted derivatives of a given haemoprotein. Magnetic data for all the derivatives studied here are available only for horse myoglobin derivatives (Scheler, Schoffa & Jung, 1957), but the spectra reported by these workers were not very different from those obtained in the present investigation. Hanania *et al.* (1966) found only minor differences between the spectra of horse and whale ferrimyoglobin derivatives.

In Fig. 1 the analysed intensity of the 18500 cm.⁻¹ band is plotted against the magnetic susceptibility at 20° as given by Scheler *et al.* (1957). Only the cyanate deviates significantly from the straight line. This compound is anomalous in other respects; Scheler *et al.* (1957) noted a linear relationship between the wavelength of the Soret band and the paramagnetic susceptibility for both myoglobin and haemoglobin derivatives, but myoglobin cyanate did not fit the straight line. The susceptibilities of

Fig. 1. Graph of the intensity of the 18500 cm.⁻¹ band against the magnetic susceptibility of myoglobin derivatives.

Table 2. *Calculated percentage of low-spin form*

Axial ligand	% of low-spin form
H ₂ O	11 (8*)
F ⁻	3 (0.7*)
H·CO ₂ ⁻	14
CH ₃ ·CO ₂ ⁻	15
NO ₂ ⁻	24
SCN ⁻	18
OCN ⁻	10
OH ⁻	35 (31*)
N ₃ ⁻	83 (79*)
CN ⁻	100 (100*)

* Estimates made by Beetlestone & George (1964) from magnetic data for horse myoglobin.

horse haemoglobin derivatives were lower than those of the corresponding horse myoglobin derivatives in all cases except the cyanate. Further magnetic studies of the myoglobin cyanate derivative are clearly needed.

The existence of a linear correlation between the magnetic susceptibility and the intensity of the 18500 cm.⁻¹ band is a strong indication that this band is indeed the β -band of the low-spin form. The straight line intersects the abscissa very close to $\chi_M(20^\circ) = 14840 \times 10^6$ c.g.s. units, the spin-only value for a sextet state. The band at 17000 cm.⁻¹ is presumed to be the corresponding α -band. The bands at 16000 and 20000 cm.⁻¹, which are characteristic of the high-spin form, are most likely to be mixed π - π^* /charge-transfer bands, as have been discussed previously (Brateman *et al.* 1964; Day *et al.* 1964). The in-plane-polarized near-infrared band (Day, Smith & Williams, 1967*a,b*) is now assigned to a pure charge-transfer transition.

We can use the data in Table 1 to determine the relative proportions of the two forms present in the magnetic mixture for each of the ten derivatives studied. The cyanide shows no trace of the high-spin bands and has a very low susceptibility. We therefore take it to be 100% low-spin. Its β -band has ϵ_{MM} 10.35 mm.⁻¹ cm.⁻¹, which we assume to be the same for all the other derivatives when in the low-spin state. The percentage of the low-spin form present is given by $100 \epsilon_{MM}(\beta)/10.35$. The calculated percentages are given in Table 2, along with the estimates of Beeston & George (1964) from magnetic data for horse myoglobins. The agreement between optically and magnetically determined percentages is quite good, although the values found by our method are consistently about 3% greater than those given by Beeston & George (1964), who assumed that the fluoride was 100% high-spin.

From the data of Tables 1 and 2 it is possible to reconstruct the spectra of the pure high-spin and low-spin forms. The results of this reconstruction for the high-spin form are given in Table 3. It is now possible to seek correlations between the positions and intensities of the two high-spin bands, whose nature is not so well understood as that of the low-spin bands.

In Fig. 2 the energies of the resolved visible bands of the high-spin form are plotted against the energy of the near-infrared band for the eight predominantly high-spin derivatives. A significant correlation is found, but the slopes of the two straight lines are less than unity. This might suggest that the near-infrared band has more charge-transfer character than the visible bands.

In Fig. 3 the position of the band at 16000 cm.⁻¹ is plotted against its intensity. The movement of the band to higher energy in the sequence $\text{CH}_3 \cdot \text{CO}_2^- \sim \text{SCN}^- < \text{H}_2\text{O} \sim \text{N}_3^- < \text{OCN}^- \sim \text{H} \cdot \text{CO}_2^- < \text{NO}_2^- < \text{F}^- < \text{OH}^-$ is closely paralleled by an increase in intensity.

The Soret band appears at about 24000 cm.⁻¹ in all the myoglobin derivatives studied and its

Table 3. Reconstructed spectra of high-spin ferrimyoglobins

Axial ligand	ν (cm. ⁻¹)	ϵ_{MM}
H_2O	15590	3.37
	19940	10.8
F^-	16590	6.74
	20310	9.58
$\text{H} \cdot \text{CO}_2^-$	15790	4.11
	19960	11.1
$\text{CH}_3 \cdot \text{CO}_2^-$	15640	2.99
	19890	11.2
NO_2^-	15820	4.42
	20230	10.0
SCN^-	15650	3.58
	19790	11.1
OCN^-	15780	4.62
	19850	9.50
OH^-	16780	6.40
	20660	12.3
N_3^-	15700	4.7

Fig. 2. Graph of band positions in the visible spectra of high-spin ferrimyoglobins against the position of the near-infrared band.

position varies from 23700 cm.⁻¹ in the cyanide derivative to 24600 cm.⁻¹ in the fluoride derivative. Scheler *et al.* (1957) showed that a linear correlation existed between the wavelength of the Soret band and the magnetic susceptibility for a number of horse myoglobin derivatives. Our analysis of the visible spectra shows that all the derivatives except the cyanide are mixtures of two spin states, so that the observed Soret band must therefore be the envelope of a Soret band of the high-spin form superimposed on that of the low-spin form. The Soret band is believed to be essentially a porphyrin π - π^* transition, and should therefore be little affected by axial co-ordination to the metal.

Fig. 3. Graph of intensity of the 16000 cm.⁻¹ band of high-spin ferrimyoglobins against position.

However, the Soret band of a high-spin haemoprotein might be expected to lie at a slightly higher energy than that of the same haemoprotein in the low-spin form, as a result of mixing with the charge-transfer band in the visible region (Brateman *et al.* 1964).

We may take the Soret band of the fluoride derivative at 24600 cm.⁻¹ as representative of the Soret band of a high-spin haemoprotein, whereas that of the cyanide derivative gives the low-spin Soret band at 23700 cm.⁻¹. The observed shift of the peak position to lower energy as the magnetic susceptibility falls can be explained by the steadily increasing contribution of the low-spin band to the overall envelope.

We can put this argument on a more quantitative basis by assuming that the high-spin and low-spin Soret bands are Gaussian, and calculating the peak position of the envelope.

A Gaussian band is represented by a function of the form:

$$\epsilon = \epsilon_0 e^{-(\nu - \nu_0)^2 / h^2}$$

ϵ_0 is the intensity maximum, ν_0 is the position of maximum intensity and h is a spreading constant. If we take the Soret bands of the fluoride and cyanide derivatives to represent the high-spin and low-spin bands respectively, we can readily construct Gaussian functions for these. By using the data of Scheler *et al.* (1957):

$$\epsilon_{\text{MM}} = 14.6e^{-(\nu - 24630)^2 / h^2} \text{ for the high-spin band}$$

$$\epsilon_{\text{MM}} = 11.6e^{-(\nu - 23700)^2 / h^2} \text{ for the low-spin band}$$

Inspection of these spectra shows that h is very close to unity in each case. Thus, in a myoglobin

Fig. 4. Theoretical curve for the variation of the Soret band position with paramagnetic susceptibility of myoglobin derivatives. Experimental points are from the data of Scheler *et al.* (1957).

derivative where the fraction of the low-spin form present is x , the ϵ_{MM} value at wave number ν will be:

$$\epsilon = (1-x)14.6e^{-(\nu - 24630)^2 / h^2} + 11.6xe^{-(\nu - 23700)^2 / h^2}$$

Differentiating with respect to ν , we get:

$$d\epsilon_{\text{MM}}/d\nu = 14.6(1-x)e^{-(\nu - 24630)^2 / h^2} \times 2(\nu - 24630) + 11.6xe^{-(\nu - 23700)^2 / h^2} \times 2(\nu - 23700)$$

At the peak position, $d\epsilon/d\nu = 0$. Then:

$$\ln \left(\frac{14.6}{11.6} \cdot \frac{1-x}{x} \right) = \ln(\nu - 23700) - \ln(\nu - 24630) + (\nu - 23700)^2 - (\nu - 24630)^2$$

$(1-x)/x$ is related to the magnetic susceptibility by the relation:

$$\frac{1-x}{x} = \frac{\chi - 1630}{14840 - \chi}$$

where χ is the susceptibility $\times 10^{-6}$ at 20° in c.g.s. units. This assumes that the paramagnetic susceptibility of the low-spin component is that of the cyanide derivative (as given by Scheler *et al.* 1957) whereas the susceptibility of the high-spin component is the spin-only value for a sextet ground state of 14840×10^{-6} c.g.s. units. We can therefore plot a theoretical graph of the susceptibility against the Soret maximum. This is shown in Fig. 4, along with the experimental points taken from the work

of Scheler *et al.* (1957). Only the imidazole derivative deviates seriously from the curve. This may indicate that the axial co-ordination of an imidazole group affects the positions of the high-spin and low-spin Soret bands. This would explain the small differences found between Soret bands of the same derivatives of different haemoproteins containing the same protoporphyrin prosthetic group. The subtle effects on the binding of the axial imidazole imposed by the conformation of the protein undoubtedly play a part in determining the specific properties of the protohaem group in different molecules.

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