Absorption Spectra of Sperm-Whale Ferrimyoglobin

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1. Sperm-whale ferrimyoglobin was found to contain 0.308% of Fe, on a dry weight basis, corresponding to a molecular weight of 18130. The solid takes up moisture to an equilibrium state, and was then assayed to contain 0.280% of Fe. 2. Absorption spectra are presented for acidic ferrimyoglobin, $\text{Fe}^+(\text{H}_2\text{O})$, and its conjugate base, $\text{Fe}-\text{OH}$, as well as for the fluoride and cyanide complexes, within the range 200–2500 μ . Data for ferromyoglobin–carbon monoxide, $\text{Fe}(\text{II})-\text{CO}$, in the visible region are also included. 3. Minor spectral differences have been found between whale and horse myoglobins, particularly in the effect of temperature on the visible-absorption spectrum of $\text{Fe}-\text{OH}$.

Comparison of the physicochemical behaviour of a protein in solution with predictions based on its known crystal structure can best be made for sperm-whale ferrimyoglobin. The X-ray diffraction studies of Kendrew *et al.* (1961) provide the necessary structural data. However, the corresponding physicochemical work on the protein in solution has been carried out mainly on ferrimyoglobin from horse-heart muscle. A systematic kinetic and thermodynamic study of the reactions of sperm-whale ferrimyoglobin in solution would be of value in two ways. It would show to what extent the reactivity of the protein accords with its known crystal structure, and would also make possible the assessment of second-order differences in the properties of myoglobins from different species with their differing basic protein structures. For such studies on solutions, detailed and reliable spectral data are needed, since much of the work involves spectrophotometric techniques. We have therefore obtained, by a standardized and independently calibrated procedure, full absorption spectra for sperm-whale ferrimyoglobin and its common derivatives.

MATERIALS AND METHODS

Sperm-whale ferrimyoglobin was purchased as a freeze-dried salt-free solid from Seravac Laboratories (Maidenhead, Berks.). This material has been shown by E. S. Awad & A. B. Edmundson (personal communication) to be chromatographically identical, on Amberlite IRC-50, with the material used by Edmundson & Hirs (1962) in their studies of amino acid sequence, and by Kendrew *et al.* (1961) in their crystallographic work. The present work was

carried out on the Seravac material without further purification.

The iron content of sperm-whale ferrimyoglobin was determined with and without prior drying. The procedure for dry weight analysis involved drying about 5 mg. of myoglobin in an oven at 105° for several hours, followed by taking the weight, W_t , of the sample on an electrobalance (Cahn Instrument Co., Paramount, Calif., U.S.A.; precision to $\pm 0.1 \mu\text{g.}$) at successive intervals of time from about $t=20$ sec. to constant 'equilibrium' weight, W_e , which is attained after 20–30 min. The dry weight was obtained by linear extrapolation of $\log(W_e - W_t)$ to zero time. Samples of the undried solid were weighed in air in the normal manner. In both cases iron determinations were made by an improved phenanthroline micro method (Cameron, 1965). By using iron-free reagents, the method was standardized by calibration against Mohr's salt, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and Oesper's salt, $\text{Fe}(\text{CH}_2\text{NH}_3)_2(\text{SO}_4)_2 \cdot 4\text{H}_2\text{O}$. The method was precise to about 1 in 300.

Absorption spectra were measured at 25° (and in some cases also at 4° and 40°), within the wavelength range 200–2500 μ with about 300 experimental points. All readings were taken in 10 mm. cells against a reagent blank. The spectra at 25° were taken on a Zeiss PMQ II spectrophotometer. At other temperatures, the spectra were taken on a Unicam SP.500 spectrophotometer in which the temperature of the cell compartment was controlled by circulating water from a thermostatically controlled bath. Concentrations of myoglobin in solution were determined with reference to the ferrimyoglobin–cyanide complex, for which the following molar extinction coefficients ($\text{M}^{-1} \text{cm.}^{-1}$) were obtained by independent duplicate iron determinations: 10.7×10^3 at 540 μ ; 110×10^3 at 423 μ .

Acidic ferrimyoglobin, $\text{Fe}^+(\text{H}_2\text{O})$, was examined at pH 6.0 and 10.02; its conjugate base, $\text{Fe}-\text{OH}$, at pH 11.5 and 10.05; the cyanide complex, $\text{Fe}-\text{CN}$, at pH 7.0 and 10.05; the fluoride complex, $\text{Fe}-\text{F}$, at pH 6.0 in 1.5 M-KF (measurements of the equilibrium constant showed that formation of the complex was 98% complete under these conditions; readings were accordingly corrected from 98 to 100% conversion into $\text{Fe}-\text{F}$). The ferromyoglobin–carbon monoxide complex, $\text{Fe}(\text{II})-\text{CO}$, was prepared by

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passing pure CO through a solution containing ferrimyoglobin in buffer at pH 7.0 with a trace of $\text{Na}_2\text{S}_2\text{O}_4$ added, and extinctions were then measured rapidly. Buffer solutions of pH 6 and 7 were 10mm- NaH_2PO_4 -NaOH mixtures, and pH 11.5 was 10mm- Na_2HPO_4 -NaOH mixture, ionic strengths being adjusted with NaCl. All reagents were of A.R. grade, and deionized glass-redistilled water was used throughout.

RESULTS AND DISCUSSION

The results of four determinations of iron in sperm-whale ferrimyoglobin are given in Table 1. The iron content is 0.308 (± 0.001)% on a dry weight basis. This corresponds to a molecular weight of 18130 and is within 2% of the value 17816 reported by Edmundson & Hirs (1962) from their amino acid analysis of the protein.

The data in Table 1 also show that when dried ferrimyoglobin is exposed to air it takes up moisture to a reproducible equilibrium state in which about 10% of water is incorporated. The iron content calculated by extrapolation to this equilibrium condition is constant at 0.280 (± 0.001)%. This latter result is also obtained, within the same limits of uncertainty, from direct iron determination on the undried stock myoglobin.

The absorption spectra of four myoglobin derivatives are shown in Figs. 1 and 2, and the main band characteristics are summarized in Table 2, which also includes data for Fe(II)-CO in the visible region of the spectrum. Several points arise on comparing these spectra of whale myoglobin with those of horse myoglobin (Hanania, 1953). As expected, their general characteristics are similar. In fact, the cyanide complexes of the two species have nearly identical spectra, which are insensitive

to changes in pH, ionic strength or temperature (from 4° to 40°). This presumably implies that strong low-spin bonding at the haematin iron is not favourable to the transmission of charge and 'allied effects' between protein and haem.

On the other hand, minor species differences are observed with the two high-spin compounds $\text{Fe}^+(\text{H}_2\text{O})$ and Fe-F . Thus whale $\text{Fe}^+(\text{H}_2\text{O})$ shows 3% stronger absorption in the visible region, and 4-5% weaker absorption in the near ultraviolet, relative to horse $\text{Fe}^+(\text{H}_2\text{O})$, and whale Fe-F is 4-9% weaker in the visible and 2% stronger at the Soret peak, the protein bands being equal. More-

Fig. 1. Absorption spectra of sperm-whale ferrimyoglobin: —, acidic ferrimyoglobin, $\text{Fe}^+(\text{H}_2\text{O})$, at 25° and at pH 6.0 and 10.02; ----, alkaline ferrimyoglobin, Fe-OH , at 25° and at pH 11.5 and 10.05.

Table 1. *Determination of iron in sperm-whale ferrimyoglobin*

Column (A), weight (mg.) of dried sample, by extrapolation; (B), weight (mg.) of dried sample after equilibration in air; (C), extinction (cm^{-1}), read at the 509mμ band against reagent blank, of the tris-(o-phenanthroline)-iron(II) complex obtained when the above sample is treated by Cameron's (1965) method and then diluted to 10ml. Concentrations of iron are calculated on the basis $\epsilon_{509 \text{ m}\mu} = 11.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

(A) mg. ($\pm 0.5\%$)	(B) mg. ($\pm 0.2\%$)	(C) Extinction ($\pm 0.2\%$)	(D) % of Fe (dry basis)	(E) % of Fe (non-dry basis)
2.340	2.569	0.142	0.308	0.281
3.154	3.509	0.192	0.309	0.278
4.570	5.012	0.277	0.308	0.281
5.549	6.138	0.337	0.308	0.279
		Mean ...	0.308	0.280

Fig. 2. Absorption spectra of sperm-whale ferrimyoglobin: —, fluoride complex, Fe-F , at 25° and at pH 7.0 in 1.5M-KF; ----, cyanide complex, Fe-CN , at 25° and at pH 7.0 and 10.05.

Table 2. *Characteristics of absorption spectra of sperm-whale ferrimyoglobin and derivatives at 25°*

max., Maximum at peak; min., minimum; sh., shoulder; ϵ ($M^{-1}cm^{-1}$), extinction coefficient.
Spectra are shown in Figs. 1 and 2.

Myoglobin derivatives									
Fe ⁺ (H ₂ O)		Fe-OH		Fe-CN		Fe-F		Fe(II)-CO	
λ (m μ)	$10^{-3} \epsilon$	λ (m μ)	$10^{-3} \epsilon$	λ (m μ)	$10^{-3} \epsilon$	λ (m μ)	$10^{-3} \epsilon$	λ (m μ)	$10^{-3} \epsilon$
252 min.	23.0	264 min.	30.0	256 min.	29.9	252 min.	27.0	495-500 min.	5.20
280 max.	31.2	281 max.	32.1	280 max.	35.0	280 max.	33.5	540 max.	14.1
290 sh.	26.7	290 sh.	28.2	290 sh.	29.0	290 sh.	27.8	558 min.	10.1
311 min.	11.6	312 min.	14.0	310 min.	14.5	310 min.	12.5	567-569 sh.	11.3
409.5 max.	157	358 sh.	33.5	348 sh.	28.0	338-340 sh.	26.2	576 max.	12.3
460 min.	7.01	414 max.	97.2	359 max.	29.4	406 max.	133		
505 max.	9.47	515 min.	7.13	379 min.	26.1	470 min.	7.30		
578 min.	2.71	542 max.	9.48	423 max.	109.7	490 max.	8.31		
590 max.	2.76	565 min.	7.65	505 min.	6.99	500 sh.	7.80		
605 min.	2.71	582 max.	9.10	540 max.	10.7	540 min.	5.27		
635 max.	3.55	595 sh.	8.38	860-880 min.	0.069	560 sh.	5.63		
715 min.	0.150	675 min.	0.421	1080-1120 sh.	0.120	580 sh.	6.37		
1040 max.	0.893	740 sh.	0.554	1280 max.	0.202	609 max.	7.82		
1370 min.	0.025	825 max.	0.754	1420 min.	0.150	~760 sh.	0.820		
1510 max.	0.409	~1000 sh.	0.14	1580 max.	0.209	860 max.	1.15		
1660 min.	0.022	1365 min.	0.003	1750 min.	0.052	1160-1380 min.	0		
> 2000 sh.	~0.60	1440 sh.	0.067	> 2000 sh.	~0.16	> 2000 sh.	~0.32		
		1520 max.	0.085						
		1660 min.	0						
		~1750 sh.	0.03						
		> 2000 sh.	~0.20						

Table 3. *Comparison of the effects of temperature on the absorption spectra of sperm-whale and horse alkaline ferrimyoglobin, Fe-OH*

Changes in extinction/degree ($\Delta E/^\circ C$) are shown at two wavelengths, and are based on a solution having $E=1.000$ at the 542m μ peak at 25°. Data for horse ferrimyoglobin are from George *et al.* (1961).

Wavelength (λ)	542m μ	582m μ
Sperm-whale ferrimyoglobin (range 4-39°)	2.4	1.8
Horse ferrimyoglobin (range 5-35°)	1.0	0.5

over, both compounds exhibit pH effects within the range pH 6-7; in the whale compounds this appears as a 2½% fall in absorption at the Soret peak, but in the horse compounds a haem-linked ionization could be inferred only indirectly from the variation of rate and equilibrium constants with pH (George & Hanania, 1955). A similar pH effect on the spectrum of Fe-F appears as a 10% change at the Soret peak in whale Fe-F and a smaller change in horse Fe-F. The spectra for both whale and horse Fe⁺(H₂O) and Fe-F are substantially unaffected by a temperature change from 4° to 40°.

With Fe-OH, the contrast between species is more marked, particularly in the visible region. Thus the characteristic double band in horse alkaline ferrimyoglobin (585 and 594m μ) appears as a band and shoulder in whale alkaline ferrimyoglobin with about 7% stronger absorption in the latter. In this region the effect of temperature is also significant. The comparative data in Table 3 show that with sperm-whale Fe-OH the effect of temperature is more than twice as large as it is with horse Fe-OH. George, Beestlestone & Griffith (1961) have shown from a consideration of the magnetic and spectral properties of horse Fe-OH that the compound is an equilibrium mixture of 70% of high-spin and 30% of low-spin electronic states, and that the transition from high-spin to low-spin bonding is slightly exothermic. No corresponding magnetic data are available for whale Fe-OH, but since the effect of temperature on its spectrum is greater than for horse Fe-OH it may be concluded that a similar electronic transition in whale Fe-OH would be expected to be more exothermic.

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