Camel Myoglobin

By E. S. AWAD AND L. KOTITE

Department of Chemistry, American University of Beirut, Beirut, Lebanon

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1. Crystalline myoglobin was prepared from camel heart muscle. 2. A method was developed for the isolation of myoglobin that employs molecular-sieve chromatography. 3. Analytical chromatography of the camel myoglobin on a molecular-sieve column and on two types of ion-exchange columns gave in each case a single elution band, which accounted for better than 98% recovery and showed that the product was free from haemoglobin. 4. The iron content on a dry weight basis was 0.308%. This value corresponds to a molecular weight of 18100. 5. The spectra of acidic ferrimyoglobin, basic ferrimyoglobin and ferrimyoglobin cyanide were measured. 6. The pK_a of the dissociation of the haem-bound water molecule in acidic ferrimyoglobin was 8.53 at 25°. 7. Conclusions are drawn about the charge on the surface of the camel ferrimyoglobin molecule as compared with horse and sperm-whale ferrimyoglobins.

The procedures that have been used by different investigators for the preparation of crystalline myoglobin vary in details, but in principle consist in extraction of myoglobin from the minced muscle with water and subsequent fractionation with ammonium sulphate between 80% and 100% saturation at pH close to 7.

Crystalline myoglobin has been prepared from horse (Theorell, 1932; Roche & Vieil, 1940; Kendrew, 1950; Lawrie, 1951; Kendrew & Trotter, 1954), cow (Roche, Derrien & Vieil, 1942; Rossi-Fanelli, Cavallini & De Marco, 1954; Lewis & Schweigert, 1955), man (Drabkin, 1945; Theorell & de Duve, 1947; Rossi-Fanelli, 1948; Rossi-Fanelli *et al.* 1954; Luginbuhl, 1960), whale (Keilin & Schmid, 1948; Kendrew, Parrish, Marrack & Orlans, 1954; Kendrew & Pauling, 1956; Kendrew & Parrish, 1957; Bradley, 1959), sheep (Beznak, 1948), turtle (Renard, 1953), carp (Hamoir, 1953), seal, penguin, porpoise, California sea lion and dolphin (Kendrew *et al.* 1954), tunny (Huys, 1954; Rossi-Fanelli & Antonini, 1955; Matsuura & Hashimoto, 1955) and molluscs (Rossi-Fanelli, Antonini & Povoledo, 1957).

Haemoglobin is a natural contaminant in the preparation of myoglobin. After the discovery that haemoglobin is insoluble whereas myoglobin is soluble in 3M-phosphate at pH 6.6 (Morgan, 1936), this difference in solubility has been used for the purification of myoglobin. Myoglobin can be separated conveniently from haemoglobin by chromatography on molecular-sieve columns (Awad, Cameron & Kotite, 1963).

Myoglobin has in several instances been shown

to be microheterogeneous. For example, sub-components of myoglobins of the following species have been resolved by ion-exchange chromatography or by electrophoresis: horse (Boardman & Adair, 1956; Åkeson & Theorell, 1960; Reichlin, Hay & Levine, 1963), man (Rossi-Fanelli & Antonini, 1957; Perkoff, Hill, Brown & Tyler, 1962), seal (Rumen, 1959), tunny (Brown, 1961) and sperm whale (Edmundson & Hirs, 1962; Atassi, 1964).

The present work describes the isolation of myoglobin from camel (*Camelus dromedarius*) heart muscle by using molecular-sieve chromatography. The product, ferrimyoglobin, was crystallized from ammonium sulphate solution and some of its physical properties were investigated.

MATERIALS

Whatman CM70 powder (CM-cellulose cation-exchanger) was obtained from W. and R. Balston Ltd., London.

Sephadex G-75 [bead form, fine grade (polydextran molecular sieve of nominal exclusion limit 50000 mol.wt.)], CM-Sephadex C-50 [fine grade (carboxymethyl-polydextran cation-exchanger)] and SE-Sephadex C-50 [fine grade (sulphonylethyl-polydextran cation-exchanger)] were obtained from Pharmacia, Uppsala, Sweden.

Sperm-whale ferrimyoglobin and horse ferrimyoglobin were obtained as salt-free freeze-dried powders from Seravac Laboratories, Maidenhead, Berks.

All other chemicals were of A.R. grade and distilled deionized water was used throughout.

METHODS

Extinction was measured with a Zeiss PMQII spectrophotometer. Silica cells of 1 cm. light-path were used.

Measurement of pH was made with a Radiometer model 4 pH-meter, with glass and calomel electrodes. The instrument was standardized at 25° against phosphate buffer, pH 6.865 (Bates, 1964).

Centrifugation was performed at 4° in a refrigerated MSE centrifuge.

A Cahn electro-microbalance was used for weighing milligram quantities of myoglobin for iron analysis.

All operations in the preparation of myoglobin were carried out in a cold room at 4°.

Molecular-sieve and ion-exchange chromatography were carried out in a cold room at 4° by using standard techniques (Boardman & Partridge, 1955; Porath, 1962).

Iron analysis was performed on 2–8 mg. samples of myoglobin, dried at 110°, by digestion in an $\text{HClO}_4\text{--H}_2\text{O}_2$ medium, reduction of the iron with hydroxylamine hydrochloride and colorimetric assay with *o*-phenanthroline (Cameron, 1965; Hanania, Yeghiayan & Cameron, 1966).

Myoglobin spectra were measured as follows. A stock solution was prepared by dissolving 80 mg. of salt-free freeze-dried myoglobin in 5 ml. of distilled water and filtering. (No significant amount of sediment remained on the filter paper, which indicated the absence of denatured material.) (a) Acidic ferrimyoglobin spectrum. A 1.00 ml. portion of the stock solution of myoglobin was diluted to 10.0 ml. with 0.05 M-phosphate buffer, pH 6.2, and the extinction was measured at wavelengths between 450 and 700 m μ against a buffer blank. This solution was diluted further, tenfold, with the same buffer and the extinction was measured at wavelengths between 240 and 450 m μ . (b) Basic ferrimyoglobin spectrum. The procedure was the same as that used for acidic ferrimyoglobin, except that the buffer was 0.05 M-borate, pH 10.8. (c) Ferrimyoglobin cyanide spectrum. Again the procedure was the same, except for the buffer, which was 0.05 M-phosphate, pH 7.0, containing 0.05% of KCN. For each spectrum, over the range 240–700 m μ , the extinction was measured at about 90 different wavelengths; near the absorption peaks, measurements were made at 2 m μ intervals. The iron content of the stock solution of myoglobin was determined as follows. A 0.300 ml. portion was transferred to a 10 ml. volumetric flask and evaporated to near-dryness on a steam bath. The procedure of Cameron (1965) was then used for the digestion of the myoglobin and the colorimetric assay of iron. The molar extinction coefficients, ϵ (M⁻¹ cm.⁻¹), were calculated on the basis of an Fe content of 0.308% and 1 Fe atom/mol. of myoglobin (i.e. a molecular weight of 18100) (see the Results section).

RESULTS

Preparation of camel myoglobin. Camel hearts were obtained immediately after slaughter and kept in ice. The muscle was dissected free from fatty tissue and ligaments, washed with tap water, drained and stored frozen at -15° for up to 10 months. Before use the muscle was thawed overnight at 4°. All operations in the preparation of the myoglobin were carried out in a cold room at 4°.

Step (1): extraction. A 3 kg. sample of camel heart muscle was cut into slices, minced in a meat grinder, suspended in 3 l. of ice-cold distilled water and

stirred slowly for 24 hr. in the cold. The mixture was squeezed through cheese-cloth, centrifuged at 3000 rev./min. (2400 g) for 2 hr. and filtered through Whatman no. 1 fluted filter paper. The pH of the filtrate was 6.4.

Step (2): concentration of myoglobin solution. The filtrate was dialysed extensively against distilled water and twice against 0.01 M-phosphate buffer, pH 6.2, and then filtered to remove a small amount of sediment. The filtrate (3430 ml.) was allowed to percolate through a Whatman CM70 (CM-cellulose) column (2.5 cm. diam. $\times$ 30 cm.) pre-equilibrated with 0.01 M-phosphate buffer, pH 6.2. In this operation the myoglobin and some haemoglobin were adsorbed in a band at the top of the column. After a brief washing with 0.01 M-phosphate buffer, pH 6.2, the band containing myoglobin was eluted from the column with 0.1 M-phosphate buffer, pH 7.3. A cut of the effluent (290 ml.) that contained the myoglobin was taken. About tenfold concentration of the original myoglobin extract was thereby achieved. The E_{410}/E_{280} extinction ratio was 1.92.

Step (3): chromatography on Sephadex G-75. The cut containing myoglobin was applied to a column (5 cm. diam. $\times$ 80 cm.) of Sephadex G-75, pre-equilibrated with 0.1 M-phosphate buffer, pH 7.3, and was eluted with the same buffer. The effluent was collected in 15 ml. fractions. The extinction of each fraction was measured at 280 and 410 m μ . When the effluent was too concentrated to be read in the spectrophotometer, a suitable dilution was made, e.g. 0.10 ml. to 5.0 ml. The elution profile is shown in Fig. 1. A cut (315 ml.) was taken by pooling the effluent fractions as indicated in Fig. 1. The E_{410}/E_{280} extinction ratio of the pooled fractions was 4.45.

Step (4): second-cycle chromatography. Steps (2), (3) and again (2) were repeated in succession. A dark-brown solution (67 ml.) of ferrimyoglobin in 0.1 M-phosphate buffer, pH 7.3, was obtained. The E_{410}/E_{280} extinction ratio was 5.08.

Step (5): freeze-drying. The solution of myoglobin was dialysed exhaustively against distilled deionized water and freeze-dried. The fluffy light-brown product (0.79 g.) was stored at 4°.

Crystallization. Suitable conditions for the crystallization of camel myoglobin were determined by setting up 30 small tubes capped tightly and containing 2 ml. of solutions of myoglobin in 75–85% saturated ammonium sulphate buffered with phosphate at pH 6.8. The concentrations of myoglobin, ammonium sulphate and phosphate were varied so that crystallization was attempted under ten different conditions. The tubes were allowed to stand at room temperature (25°) without being disturbed. Crystallization took place within 4 weeks in most of the tubes. The following

Fig. 1. Elution profile obtained in step (3) in the preparation of camel myoglobin (see the text). A Sephadex G-75 column (5 cm. diam. $\times$ 80 cm.) was used, the eluent being 0.1 M-phosphate buffer, pH 7.3.

Fig. 2. Camel ferrimyoglobin crystals. Magnification $\times$ 50.

procedure was used to crystallize a large amount of myoglobin.

A filtered solution of 0.5 g. of salt-free freeze-dried myoglobin dissolved in 30 ml. of distilled water was mixed with a filtered solution of saturated ammonium sulphate-2 M-phosphate buffer, pH 6.8 (19:1, v/v). A suspension (1 ml.) containing seed crystals was added. The mixture was kept in a glass-stoppered flask at 25° in a thermostatically controlled bath. Crystallization was complete within 4 days.

Fig. 3. Chromatographic analysis of camel ferrimyoglobin. (a) Molecular-sieve chromatography on a Sephadex G-75 column (2.5 cm. diam. $\times$ 34 cm.), the eluent being 0.05 M-phosphate buffer, pH 6.84, and the flow rate 8.4 ml./hr.; the sample contained 40 mg. in 1 ml. (b) Ion-exchange chromatography on a CM-Sephadex C-50 column (2.5 cm. diam. $\times$ 29 cm.), the eluent being 0.05 M-phosphate buffer, pH 6.85, and the flow rate 9.6 ml./hr.; the sample contained 40 mg. in 1 ml. (c) Ion-exchange chromatography on an SE-Sephadex C-50 column (2.5 cm. diam. $\times$ 28 cm.), the eluent being 0.05 M-phosphate buffer, pH 6.82, and the flow rate 14 ml./hr.; the sample contained 20 mg. in 1 ml.

The crystals were dark brown, the largest approx. 2 mm. $\times$ 0.5 mm. $\times$ 0.02 mm. A photograph is shown in Fig. 2.

Analytical chromatography. Samples of camel ferrimyoglobin were analysed by molecular-sieve chromatography on Sephadex G-75, the eluent being 0.05 M-phosphate buffer, pH 6.84, and by ion-exchange chromatography on CM-Sephadex C-50, the eluent being 0.05 M-phosphate buffer, pH 6.85, and on SE-Sephadex C-50, the eluent being 0.05 M-phosphate buffer, pH 6.82. Elution profiles are shown in Fig. 3. In each of the three chromatographic systems, a single elution band was obtained which accounted for at least 98% recovery. Other runs on columns of SE-Sephadex C-50, the eluents being 0.05 M-phosphate buffer at pH 6.40 and at pH 7.42, gave similar elution profiles. These results show that no significant amounts of haemoglobin were present, since both the molecular-sieve and ion-exchange chromatography would have resolved any haemoglobin component in the sample. Comparison of the behaviour of camel ferrimyoglobin with horse and sperm-whale ferrimyoglobins in the same ion-exchange chromatographic system indicates that camel and horse ferrimyoglobins are about equally positively charged at pH 6.85, whereas sperm-whale ferrimyoglobin is considerably more positively charged. This conclusion is borne out by the data in Table 1.

Table 1. *Elution volumes of ferrimyoglobin of different species chromatographed on CM-Sephadex C-50 with 0.05 M-phosphate buffer, pH 6.85*

Samples (40 mg.) of myoglobin dissolved in 1.0 ml. of buffer were applied to the column (2.5 cm. diam. $\times$ 29 cm.) of CM-Sephadex C-50. The eluent was 0.05 M-phosphate buffer, pH 6.85, the flow rate being 9.6 ml./hr.

	Elution volume (ml.)
Camel ferrimyoglobin	108
Horse ferrimyoglobin (major component*)	129
Sperm-whale ferrimyoglobin (major component*)	520

* Horse and sperm-whale ferrimyoglobins were resolved into one major and several minor components in this chromatographic system (E. S. Awad & L. Kotite, unpublished work). Edmundson & Hirs (1962) have resolved sperm-whale myoglobin into five components on Amberlite IRC-50 columns with 0.34 N-sodium citrate-NaCl buffer, pH 5.88.

The analytical chromatography on CM-Sephadex C-50 and on SE-Sephadex C-50 indicates that the sample of camel ferrimyoglobin was microhomogeneous. Sperm-whale and horse ferrimyoglobins were each resolved into at least four sub-components in the same chromatographic systems.

Iron content and molecular weight. Iron analysis was performed on six samples of myoglobin. The analytical results gave an average value of $0.308 \pm 0.002\%$ of iron, which yields a calculated molecular weight of 18 100 on the basis of 1 atom of iron/mol. of myoglobin. The molecular-sieve elution volume, which was obtained in the runs described above, precludes any integral multiple of this molecular weight.

Spectra. The spectra of acidic ferrimyoglobin, basic ferrimyoglobin and ferrimyoglobin cyanide are shown in Fig. 4. Values of the molar extinction coefficients at the maxima, minima and shoulder regions of the spectra are given in Table 2.

Acid dissociation of the haem-bound water molecule. Stock ferrimyoglobin solution (approx. 1 mM) was prepared by dissolving 100 mg. of salt-free freeze-dried myoglobin in 15 ml. of distilled water, adding a few small crystals of sodium nitrite, dialysing twice for 3 hr. against distilled water to remove excess of nitrite and filtering. Of this solution 1.00 ml. was added to 9.00 ml. of buffer of ionic strength 0.05 (phosphate buffer, pH 6.2, glycine buffer, pH 11.0, and ten borate buffers varying in pH between 8.3 and 10.6). The pH of the mixture was measured with an accuracy of 0.002 pH unit and the extinction was determined at 408 m μ , which is the wavelength of maximum

Fig. 4. Spectra of camel myoglobin: *A*, acidic ferrimyoglobin in 0.05 M-phosphate buffer, pH 6.2; *B*, basic ferrimyoglobin in 0.05 M-borate buffer, pH 10.8; *C*, ferrimyoglobin cyanide in 0.05 M-phosphate buffer, pH 7.0, containing 0.05% of KCN. The spectra were measured at room temperature (25°). Molar extinction coefficients are given in Table 2.

difference in the extinction between the acidic and basic forms of camel ferrimyoglobin.

It was assumed that ferrimyoglobin in the phosphate buffer (pH 6.2) is entirely in the acidic form, i.e. $\text{Fe}(\text{H}_2\text{O})^+$, and in the glycine buffer (pH 11.0) entirely in the basic form, i.e. FeOH , whereas in the borate buffers there exists a mixture of acidic and basic forms. The degree of dissociation, α , of the haem-bound water molecule, which corresponds to the equilibrium:

is given by:

$$\alpha = [\text{FeOH}] / ([\text{Fe}(\text{H}_2\text{O})^+] + [\text{FeOH}]) \\ = (E_{\text{borate}} - E_{\text{glycine}}) / (E_{\text{phosphate}} - E_{\text{glycine}})$$

where E is the extinction of the myoglobin solution at 408 m μ and the subscript refers to the buffer in which the extinction was measured. Defining the acid dissociation constant, K_a , by the equation:

$$K_a = [\text{FeOH}][\text{H}^+] / [\text{Fe}(\text{H}_2\text{O})^+]$$

then:

$$K_a = \alpha[\text{H}^+] / (1 - \alpha)$$

where $[\text{H}^+]$ is the activity of H^+ ion as measured by the glass electrode. Rearrangement of the last equation gives:

$$\alpha = 1 - \alpha[\text{H}^+] / K_a$$

It can be shown that K_a is given by the intercept on the abscissa of a plot of α against $\alpha[\text{H}^+]$. A typical plot is shown in Fig. 5. The results of four runs gave an average $\text{p}K_a$ value of 8.53 at 25° (average deviation less than ± 0.005).

Table 2. *Spectral characteristic of camel myoglobin*

The spectra are given in Fig. 4. ϵ , Molar extinction coefficient ($\text{M}^{-1}\text{cm}^{-1}$); (max.), maximum; (min.), minimum; (sh.), shoulder.

Acidic ferrimyoglobin		Basic ferrimyoglobin		Ferrimyoglobin cyanide	
λ (m μ)	$10^{-3}\epsilon$	λ (m μ)	$10^{-3}\epsilon$	λ (m μ)	$10^{-3}\epsilon$
250 (min.)	24.1	259 (min.)	30.7	257 (min.)	27.7
274 (sh.)	30.8	274 (sh.)	33.6	274 (sh.)	32.7
280 (max.)	31.3	280 (max.)	34.3	280 (max.)	33.3
290 (sh.)	26.6	290 (sh.)	29.3	290 (sh.)	28.1
310 (min.)	12.4	308 (min.)	13.7	308 (min.)	13.7
360 (sh.)	33.0	360 (sh.)	36.2	360 (max.)	31.4
409 (max.)	172.0	414 (max.)	104.0	378 (min.)	28.7
466 (min.)	7.94	480 (sh.)	10.20	423 (max.)	115.0
470 (max.)	7.96	512 (min.)	7.83	505 (min.)	7.45
474 (min.)	7.94	542 (max.)	9.48	542 (max.)	11.32
503 (max.)	9.87	562 (min.)	7.93	570 (sh.)	8.25
573 (min.)	3.55	587 (max.)	9.30		
580 (max.)	3.60	600 (sh.)	8.65		
605 (min.)	3.10	680 (min.)	0.57		
630 (max.)	3.66				
$\epsilon_{410}/\epsilon_{280} = 5.49$		$\epsilon_{414}/\epsilon_{280} = 3.03$		$\epsilon_{423}/\epsilon_{280} = 3.46$	

Fig. 5. Example of plot for the determination of pK_a of the haem-bound water molecule in camel acidic ferrimyoglobin (see the text). The intercept on the abscissa gives K_a 2.88×10^{-9} , i.e. pK_a 8.54.

DISCUSSION

The main advantage of the present procedure for the preparation of myoglobin is the ease and thoroughness with which haemoglobin is removed by use of a molecular-sieve column. Other proteins are either not extracted from the muscle with distilled water in step (1) of the preparation, or are not adsorbed on Whatman CM-cellulose in step (2) and are therefore washed out of the column, or are separated out by the molecular-sieve column in step (3). The identity and purity of the product are established by the spectra, the iron analysis and the analytical chromatography.

The molar extinction coefficient of the protein

band in camel acidic ferrimyoglobin, $\epsilon_{280} 31.3 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$, is very close to the corresponding values for horse acidic ferrimyoglobin ($\epsilon_{280} 31.3 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$; G. I. H. Hanania, personal communication) and for sperm-whale acidic ferrimyoglobin ($\epsilon_{280} 31.2 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$; Hanania *et al.* 1966). The E_{409}/E_{280} extinction ratio for camel acidic ferrimyoglobin is 5.49, higher than the corresponding values of 5.19 for horse acidic ferrimyoglobin (G. I. H. Hanania, personal communication) and 5.03 for sperm-whale acidic ferrimyoglobin (Hanania *et al.* 1966). This indicates that the electronic distribution in the region of the haem group is different in the myoglobins of these three species.

The pK_a for the ionization of the haem-bound water molecule in camel acidic ferrimyoglobin is 8.53, lower than that for horse acidic ferrimyoglobin (pK_a 8.92 at 20° and 10.08; George & Hanania, 1952) and that for sperm-whale acidic ferrimyoglobin (pK_a 8.96 at 25° and 10.05; G. I. H. Hanania, personal communication). The difference of 0.4 pH unit is beyond experimental error. As a check, we have measured the pK_a values for horse acidic ferrimyoglobin and for sperm-whale acidic ferrimyoglobin, using the same buffer solutions as were used in our measurements of the pK_a of camel acidic ferrimyoglobin. For horse acidic ferrimyoglobin we obtained pK_a 8.94 and for sperm-whale acidic ferrimyoglobin pK_a 8.97, in good agreement with the values of Hanania and co-workers. The comparatively low value of the pK_a of the ionization of the water molecule in camel ferrimyoglobin indicates that the local ionic

environment of the haem group in camel ferri-myoglobin, at about pH 8.5, is more positive than in horse and sperm-whale ferrimyoglobins.

In contrast, camel and horse ferrimyoglobins, at about pH 7, have a smaller overall positive surface charge than sperm-whale ferrimyoglobin, as evidenced by the ion-exchange chromatography.

Such species differences are not unexpected, since there is good reason to believe that the amino acid composition and sequence are not identical for myoglobins of different species.

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