Chromatography of Myoglobin on Diethylaminoethyl Cellulose Columns*

W. Duane Brown

From the Institute of Marine Resources, University of California, Berkeley, California

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Since the initial description (2, 3) of procedures for the use of cellulose ion exchange columns for the chromatography of proteins, a number of such systems have been developed for specific proteins, including the heme proteins. Thus, Gutter et al. (4) have described the use of carboxymethyl cellulose in a study of various globulins. Ramon (5) has utilized carboxymethyl cellulose for the identification of multiple myoglobins from the seal and Bartech and Kamen (6) have used diethylaminoethyl cellulose for the isolation of heme proteins from Chromatium. Since the completion of the work described herein, Akeson et al. (7) and Akeson and Theorell (8) have described the use of carboxymethyl cellulose columns for the preparation of myoglobins and isolation of myoglobin from muscle. This report describes procedures for the chromatography of myoglobins on diethylaminoethyl cellulose columns that are suitable for quantitative determination of myoglobin in extracts, myoglobin-hemoglobin differentiation, and isolation of myoglobin from muscle.

EXPERIMENTAL PROCEDURE

Materials—DEAE-cellulose was purchased from Bio Rad Laboratories, Richmond, California. Different lots had exchange capacities of 0.6 to 0.7 meq per g. Tris (primary standard grade) was obtained from Sigma Chemical Company.

Myoglobins were isolated from the red skeletal muscle of yellowfin tuna (Nelkunnnus macropeterus), albacore tuna (Thunnus germo) and whale (Megaptera nodosa) by repeated ammonium sulfate fractionation. Details of this procedure and sources of the raw materials have been described previously (9, 10). A sample of myoglobin was similarly prepared from bovine muscle. Albacore tuna, whale, and human hemoglobins were prepared by ammonium sulfate fractionation of extracts of washed lysed red blood cells. Other hemoglobins were Mann products.

Myoglobins were in the met-(ferri-) state after the isolation procedure. For conversion to carboxymyoglobin, a solution of metmyoglobin was reduced with a few crystals of sodium dithionite, and CO gas was bubbled through. The completeness of the conversion was checked spectrophotometrically.

Preparative Procedures with DEAE-cellulose Chromatography—Samples of bluefin tuna (Thunnus thynnus) and Chinook salmon (Oncorhynchus tsawytscha) hearts and cormorant (Phalacrocorax) muscle were used. The tuna hearts were obtained from the F. E. Booth Company, San Francisco, and the salmon hearts were supplied by Mr. Alvin Jensen of the Salmon Nutrition Laboratory, Cook, Washington. The cormorant tissue was supplied by Drs. A. L. Tappel and T. Richardson, who obtained it through the cooperation of the California Fish and Game Commission. All tissues were frozen after collection and kept frozen until the start of myoglobin isolation procedures. An aliquot of tissue was thawed, minced, and homogenized with 1 volume of distilled water; the mixture was then centrifuged in a Servall angle head refrigerated centrifuge (2°C) for 10 minutes at 5000 × g. With the hearts, the tissues were rinsed with distilled water before blending to remove excess hemoglobin. The supernatant from the centrifugation was filtered through cheesecloth to remove fatty material. A convenient but not necessary step at this point was to dialyze the supernatant against a few changes of distilled water; this resulted in the precipitation of some of the extraneous proteins. If this step was included, the supernatant was again centrifuged and the precipitate discarded. The supernatant was then brought to 50% saturation with ammonium sulfate, and the pH was adjusted to 6.8 with ammonium hydroxide. The precipitate at 50% saturation was discarded, and the ammonium sulfate concentration was increased in increments of 10% with centrifugal separation of the resulting precipitates until the myoglobin fraction precipitated. This was ordinarily at about 80% saturation or greater but varied depending on the source; for example, cormorant myoglobin did not precipitate until the solution was nearly saturated with ammonium sulfate (about 95%). The precipitate was collected, dissolved in a small volume of water, and dialyzed in the cold against several changes of ion-free water until completely free of ammonium sulfate and was then dialyzed against the buffer to be used for the chromatography as described below.

Chromatography—The cellulose packing material was washed two or three times with water and then washed repeatedly with buffer until pH equilibrium was attained. For preliminary studies and microquantitative work, 0.5- × 7-cm columns were used, whereas 2.0- × 6-cm columns were used for recovery determinations with larger amounts of material as well as for smaller scale preparative work. For larger scale preparations, columns 4.5 × 8 or 18 cm were used. The small columns were packed by gravity flow, whereas the 2.0- × 6-cm columns were packed under 0.5 or 1 pound of air pressure and the 4.5- × 8- or 18-cm columns were packed under 2 pounds of air pressure. Flow rate on the smallest columns was about 1 ml per 6 minutes. Size of the collected fraction varied depending on the purpose of the column but was usually about 0.2 to 0.5 ml. The 2- × 6-cm column rate under gravity flow varied with buffer and load conditions but was about 10 to 20 ml per hour with 1 to 5 ml samples being collected. The largest columns (4.5 × 8 or 18 cm) were packed under 2 pounds of air pressure and were usually developed under 1 pound of pressure. Samples (0.1 to 1.0 ml) applied to columns were usually collected in 2-ml fractions in the small columns and 5-ml fractions in the larger columns. Four to eight changes of distilled water were used to elute myoglobin from the columns, and the eluate was collected in 2-ml fractions. The pH was adjusted to 6.0 or 6.5 with ammonium hydroxide and dialyzed at 0°C against several changes of ion-free water; this resulted in the precipitation of some of the extraneous proteins. The supernatant was then brought to 50% saturation with ammonium sulfate, and the pH was adjusted to 6.8 with ammonium hydroxide. The precipitate at 50% saturation was discarded, and the ammonium sulfate concentration was increased in increments of 10% with centrifugal separation of the resulting precipitates until the myoglobin fraction precipitated. This was ordinarily at about 80% saturation or greater but varied depending on the source; for example, cormorant myoglobin did not precipitate until the solution was nearly saturated with ammonium sulfate (about 95%). The precipitate was collected, dissolved in a small volume of water, and dialyzed in the cold against several changes of ion-free water until completely free of ammonium sulfate and was then dialyzed against the buffer to be used for the chromatography as described below.

* A preliminary report of this work has appeared (1).
Thus, in these cases, only the visible region was used. The peak is meaningless as an assay for samples containing dithionite. However, the 
280-nm solutions absorb strongly in the ultraviolet region, the 280-nm metric assay were done with the same buffer. Because dithionite derivatives are: myoglobin 56 (maximum at 431 nm), carboxy- 
globin. For example, values of ~1% for bluefin tuna myoglobin through to ensure complete conversion to the carboxymyoglobin 
appearance of the whale and tuna myoglobins in which we were primarily interested, a majority of the columns were run at room temperature. Myoglobins were always eluted starting with the same buffer with which the column was equili-
bated (Tris, pH 8.2 to 8.6, 0.005 to 0.05 M); hemoglobins and other soluble proteins were eluted by a change to buffers of lower pH or higher ionic strength (usually 0.02 to 0.2 M NaCl) as indicated in the results section. The change in buffers on the column was made directly. 

Analysis of Effluent Fractions—The concentration of proteins in the effluent fractions was assayed by measurement of the Soret absorption peak (406 to 423 nm depending on derivative and buffer), the ultraviolet absorption in the 280-nm region in a Cary model 11 recording spectrophotometer, or both. The entire Soret peak was recorded and the maximum taken; this is important for quantitative work because maxima for hemoglobin and myoglobin may differ slightly. Also, at pH 8.6 the absorption maximum of metmyoglobin is slightly different from that in aqueous solution at neutral pH. Metmyoglobin was unchanged on the columns under the conditions of this study but the carboxymyoglobin may undergo slight dissociation during passage through the column. Therefore, for maximal accuracy, each effluent fraction or an aliquot thereof was treated with a small (1-mg) quantity of sodium dithionite, and CO gas was bubbled through to ensure complete conversion to the carboxymyoglobin form before recording of the Soret peak. The extinction coefficient of the carboxymyoglobin Soret peak is higher than the corresponding nonsubstituted ferrous myoglobin or myoglobin. For example, values of εsmax for bluefin tuna myoglobin derivatives are: myoglobin 56 (maximum at 431 nm), carboxymyoglobin 105 (maximum at 420 nm), and metmyoglobin 86 (maximum at 407 nm). Because of the slight shifts noted in the Tris buffer at pH 8.6, all dilutions of fractions for spectrophotometric assay were done with the same buffer. Because dithionite solutions absorb strongly in the ultraviolet region, the 280-nm peak is meaningless as an assay for samples containing dithionite. Thus, in these cases, only the visible region was used.

RESULTS AND DISCUSSION

Chromatography of Purified Myoglobin—Fig. 1 illustrates the effect of varying the concentration of Tris buffer, pH 8.6, on the chromatography of purified whale myoglobin derivatives on DEAE-cellulose columns. Myoglobin is eluted near the hold-up volume at the higher buffer concentration (0.05 M), whereas appreciable retention is noted at buffer concentration of 0.01 or 0.02 M as shown. Data obtained from runs with still lower concentration of buffer show similar results; the elution volume increases with decreasing molarity of buffer. Similar data have been obtained with myoglobins from bovine muscle, albacore tuna, yellowfin tuna, bluefin tuna, salmon, and cormorant.

Measured percentage recoveries of carboxymyoglobin ranged from 88 to 100% and an average of five recoveries was 95%. Recovery of metmyoglobin from the column was nearly quantitative in every case. As shown in Fig. 1, the chromatography of myoglobin is qualitatively similar regardless of whether the carboxy- or met- form is used; metmyoglobin moves more rapidly than carboxymyoglobin during elution with buffers of lower concentrations. Use of the met- form is advantageous if it is desired to follow elution patterns by the ultraviolet absorption peak or to note the Soret to ultraviolet absorbance ratio of the protein.

Separation of Hemoglobin and Myoglobin—Fig. 2 illustrates the separation of a mixture of hemoglobin and myoglobin on a DEAE-cellulose column. There is no movement of hemoglobin under the indicated conditions until either the pH is lowered or ionic strength increased. Better elution patterns were obtained with the larger (2.0- X 6-cm) columns but the small (0.5- X 6-cm) columns gave satisfactory results and hence are suitable for preliminary studies or working with micro amounts of material. Recoveries of hemoglobin were always lower than those of myoglobin; better recoveries of hemoglobin were obtained when it was in the carboxy- rather than other forms. Separation of numerous pairs of myoglobin-hemoglobin mixtures has been accomplished with similar results.

It is possible to place an aqueous extract of muscle directly on a column and achieve a separation of myoglobin from hemoglobin, provided that the total ionic strength is not too great. The effluent myoglobin is free of hemoglobin; thus, a quantitative determination of relative percentages of the two heme proteins in an extract can be accomplished. This is conveniently done.
Chromatography of Myoglobin

FIG. 2. Chromatography of a mixture of whale carboxymyoglobin (Peak A) and bovine carboxyhemoglobin (Peak B). DEAE-cellulose column, 2.0 X 6 cm, 0.02 M Tris buffer, pH 8.6; switched to 0.02 M Tris buffer, pH 7.3, as indicated. Soret maxima: carboxymyoglobin, 423 mp; carboxyhemoglobin, 420 mp.

FIG. 3. Preparative chromatography of a bluefin tuna heart extract. DEAE-cellulose column, 4.5 X 18 cm, Tris buffer, pH 8.6, 0.05 M NaCl in the same buffer started as indicated. Peaks A and B are myoglobins. C is hemoglobin and other proteins.

as follows: an extract is clarified by high speed centrifugation and the total absorbancy in the Soret region of the extract is compared to the effluent myoglobin. Inasmuch as the presence of noncolored contaminating proteins does not interfere with such an assay, the myoglobin can be collected in a single fraction as determined by visual inspection. The application of such a technique to the light colored loin muscle of yellowfin tuna indicated that 86% of the total heme pigments was myoglobin.

Preparative Chromatography—Fig. 3 is an example of elution patterns obtained from preparative DEAE-cellulose columns. Best results with the preparative columns were obtained when the starting crude extract had been fractionated at least once by ammonium sulfate precipitation, and the resulting myoglobin-rich fraction dialyzed against starting buffer before application to the column. The ratio of the Soret to ultraviolet absorbancy in the effluent is a good indicator of the purity of the preparation. In Fig. 3 this ratio for the main myoglobin peak in Tris buffer, pH 8.0, is about 4.5, (the same as for known purified bluefin myoglobin in this buffer), whereas the ratio for the second myoglobin is about 4.0, indicating that the second myoglobin might have been slightly contaminated by other proteins. Contamination was obvious in the eluted hemoglobin fraction, where the ratio was approximately 0.95. Rechromatography of Peak A gave an elution curve similar to those in Fig. 1 with quantitative recovery. In the case of the bluefin tuna heart preparation illustrated in Fig. 3, the approximate total recovery of heme proteins from the once-precipitated extract was 90% (80% as main myoglobin fraction) and total recovery of protein was approximately 65% as estimated from ultraviolet absorbancies.

We have not attempted chromatography to separate multiple myoglobins known to occur in various animal muscle tissues (6, 8, 11); techniques have recently been described for the use of carboxymethyl cellulose for this purpose (7, 8).

SUMMARY

Methods are described for the chromatography of myoglobin on DEAE-cellulose, with Tris buffer, pH 8.2 to 8.6, and concentrations of 0.005 to 0.05 M. With 0.05 M buffer, myoglobin is eluted near the hold-up volume and is increasingly retained as the molarity of the buffer is decreased. Under the above conditions, hemoglobin is retained on the top of the column, making possible a simple differentiation between the two heme proteins as well as a determination of relative percentages of the two in tissue extracts. Hemoglobin may be removed from the column by increasing the ionic strength or decreasing the pH of the buffer. Procedures similar to those described may be used in larger columns for preparative isolation of purified myoglobin from relatively crude muscle extracts.

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REFERENCES

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