CHROM. 4015

ISOELECTRIC FRACTIONATION OF BOVINE METMYOGLOBIN*

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SUMMARY

Isoelectric fractionation was used to separate nine metmyoglobin components from partially purified bovine metmyoglobin and to determine the isoelectric point of each component. Isoelectric fractionation demonstrated that the fractions from CM-cellulose column chromatography and the bands from polyacrylamide gel electrophoresis are heterogeneous. The inability of metmyoglobin components to interconvert with one another was also demonstrated. During the isoelectric fractionation a small amount of the metmyoglobin was reduced and oxymyoglobin was formed.

INTRODUCTION

The microheterogeneity of bovine metmyoglobin (MetMb) was established and described by QUINN *et al.*[1], FELLAND AND SNYDER[2], SATTERLEE *et al.*[3] and DUFRESNE[4] using the separation techniques of CM-cellulose column chromatography, starch gel and polyacrylamide gel electrophoresis. Depending upon the separation technique employed, the number of MetMb components obtained varied. CM-cellulose chromatography could separate three MetMb fractions and starch gel could separate three MetMb bands whereas the more sensitive polyacrylamide gel electrophoresis could separate four MetMb bands. The introduction of the isoelectric fractionation technique by VESTERBERG AND SVENSSON[5] and VESTERBERG[6] made possible the separation of proteins whose isoelectric points differed by only 0.06 pH units. In this study the isoelectric fractionation technique was used to separate the components of bovine MetMb and also to investigate the heterogeneity of the MetMb fractions from CM-cellulose chromatography and the bands from polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

The MetMb samples used were isolated from bovine leg muscle following the procedure of SNYDER AND AYRES[7], except crystals were not obtained. The partially

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purified MetMb used was the fraction precipitated by more than 85 % saturation with $(NH_4)_2SO_4$.

The partially purified MetMb was further purified on CM-cellulose column chromatography. The MetMb components were eluted from the column (2.5 × 25 cm) with the aid of a linear pH gradient (pH 6.0 to 8.0) formed by pH 6.0 and pH 8.0 10 m*M* phosphate buffer. The partially purified MetMb was also further purified on polyacrylamide gel electrophoresis. The gel electrophoresis used a 5 % gel with a discontinuous buffer system consisting of 76 m*M* Tris (pH 8.4) buffer in the gel and 0.3 *M* boric acid and 0.05 *M* NaOH (pH 7.6) as the circulating buffer.

To separate a MetMb sample by isoelectric fractionation, the sample was first dialyzed and then lyophilyzed to dryness. The sample consisted of 35 mg of the lyophilyzed MetMb dissolved in 4 ml of distilled water. The isoelectric fractionation apparatus was then prepared as described by VESTERBERG AND SVENSSON[5] and VESTERBERG[6]. When the ampholytes and the sample had been placed in the column, a potential was applied to the column and gradually increased to 1000 V. This potential was maintained for 24 to 36 h to obtain complete separation of the MetMb components. The column was maintained at 4° during the experiment.

After complete separation of the MetMb components, the potential was removed from the column, and the column was emptied at a flow rate of 2.5 ml/min into a series of test tubes. The absorbance was determined for each tube at 409 nm. The pH was also determined for each tube with a Beckman Expanded Scale pH meter. The pH of the tube corresponded to the isoelectric point of the component in that tube.

Several components seen in the isoelectric fractionation column could not be seen upon elution because of diffusion occurring during the elution process. The isoelectric point of each of these components was determined by first measuring the distance the component was located from the main component, and then using the predetermined pH/distance value to obtain the isoelectric point.

Each component present on the elution pattern was collected, extensively dialyzed against distilled water and then lyophilyzed to dryness.

RESULTS AND DISCUSSION

When partially purified bovine MetMb was analyzed by isoelectric fractionation, three zones of MetMb were seen on the column. The largest MetMb zone, IF_1, was nearest the cathode and possessed the highest isoelectric point. The zone just above IF_1 was seen to consist of three components, $IF_{2,1}$, $IF_{2,2}$, and $IF_{2,3}$. The zone nearest the anode was shown to consist of three components, $IF_{3,1}$, $IF_{3,2}$, and $IF_{3,3}$. The separation obtained with partially purified MetMb is shown in Fig. 1. The resolution seen in Fig. 1 was lost when the column was emptied. The elution pattern obtained from separation of partially purified MetMb is shown in Fig. 2.

The loss in resolution upon emptying the column was believed to have been due to the diffusion of the MetMb after removing the electrical potential.

QUINN *et al.*[1], FELLAND AND SNYDER[2], SATTERLEE *et al.*[3] and ATASSI[8] observed that the MetMb fractions from the CM-cellulose chromatography of sperm whale and bovine MetMb were heterogeneous. Each fraction gave several bands when placed upon polyacrylamide gel electrophoresis or several fractions from rechromatography on CM-cellulose. In this study, the three fractions from CM-cellulose column chromato-

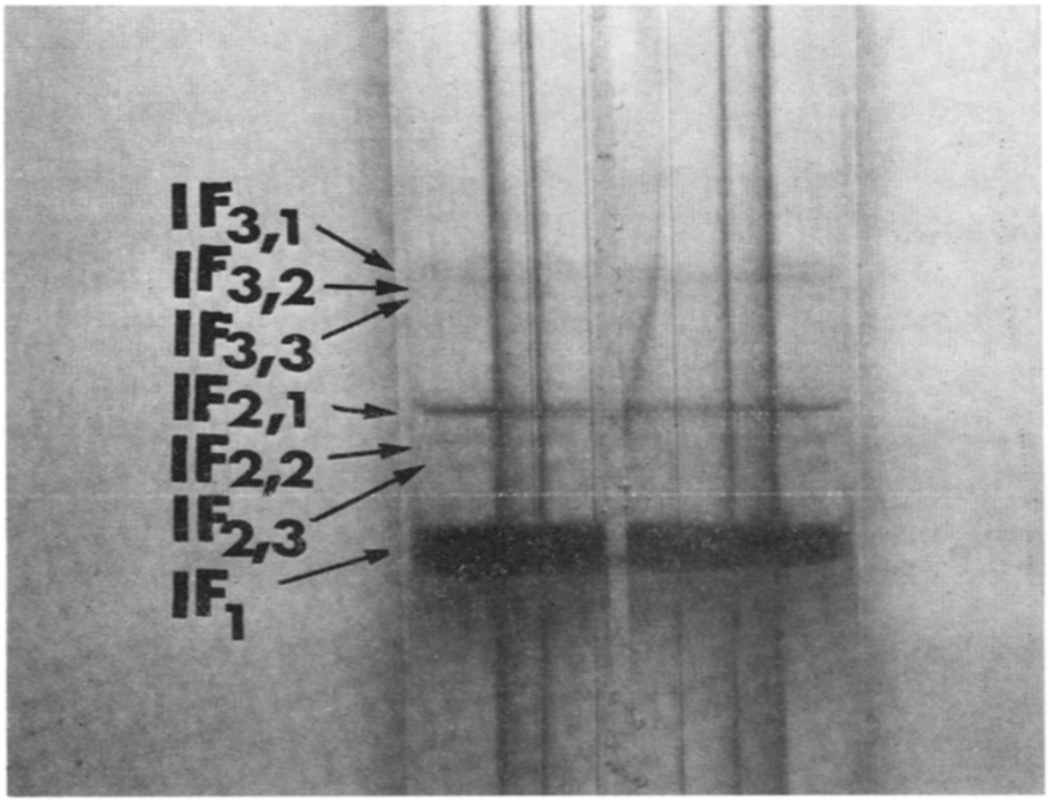

Fig. 1. The MetMb components from partially purified MetMb on pH 6 to 8 isoelectric fraction column. The sample, 28.8 mg, was separated with a 1000 V potential for 41 h. The cathode is at the bottom of the column.

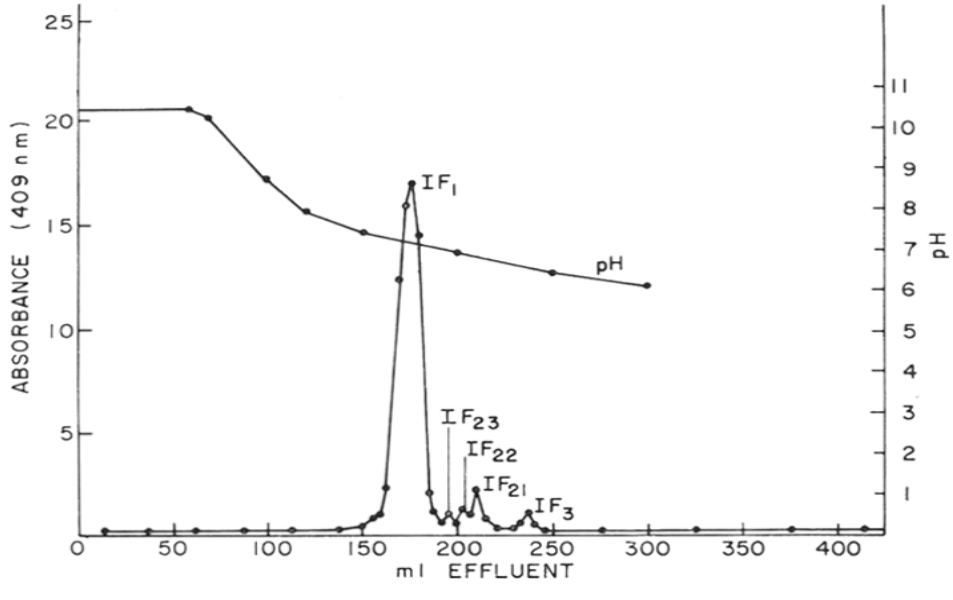

Fig. 2. The elution pattern from the pH 6 to 8 isoelectric fractionation column. The sample was partially purified MetMb.

graphy were placed on the isoelectric fractionation apparatus and the amounts of each component present in each fraction were determined. Fig. 3 shows the components present in the fractions from CM-cellulose chromatography. Fraction I is shown to contain four components, fraction II six, and the fast-moving fraction (FMF) nine, upon isoelectric fractionation. The FMF contains the largest amounts of the minor MetMb components. This fraction contains the components shown in Fig. 1 and also a fourth zone of components just above the IF_3 zone. This zone, IF_4, is composed of two components, $IF_{4,1}$ and $IF_{4,2}$. These components are the most negative components since they lie nearest the anode and possess the lowest isoelectric points.

The $IF_{4,1}$ and $IF_{4,2}$ components are seen in the FMF and not in the partially purified MetMb because of the higher concentration of minor components in the FMF.

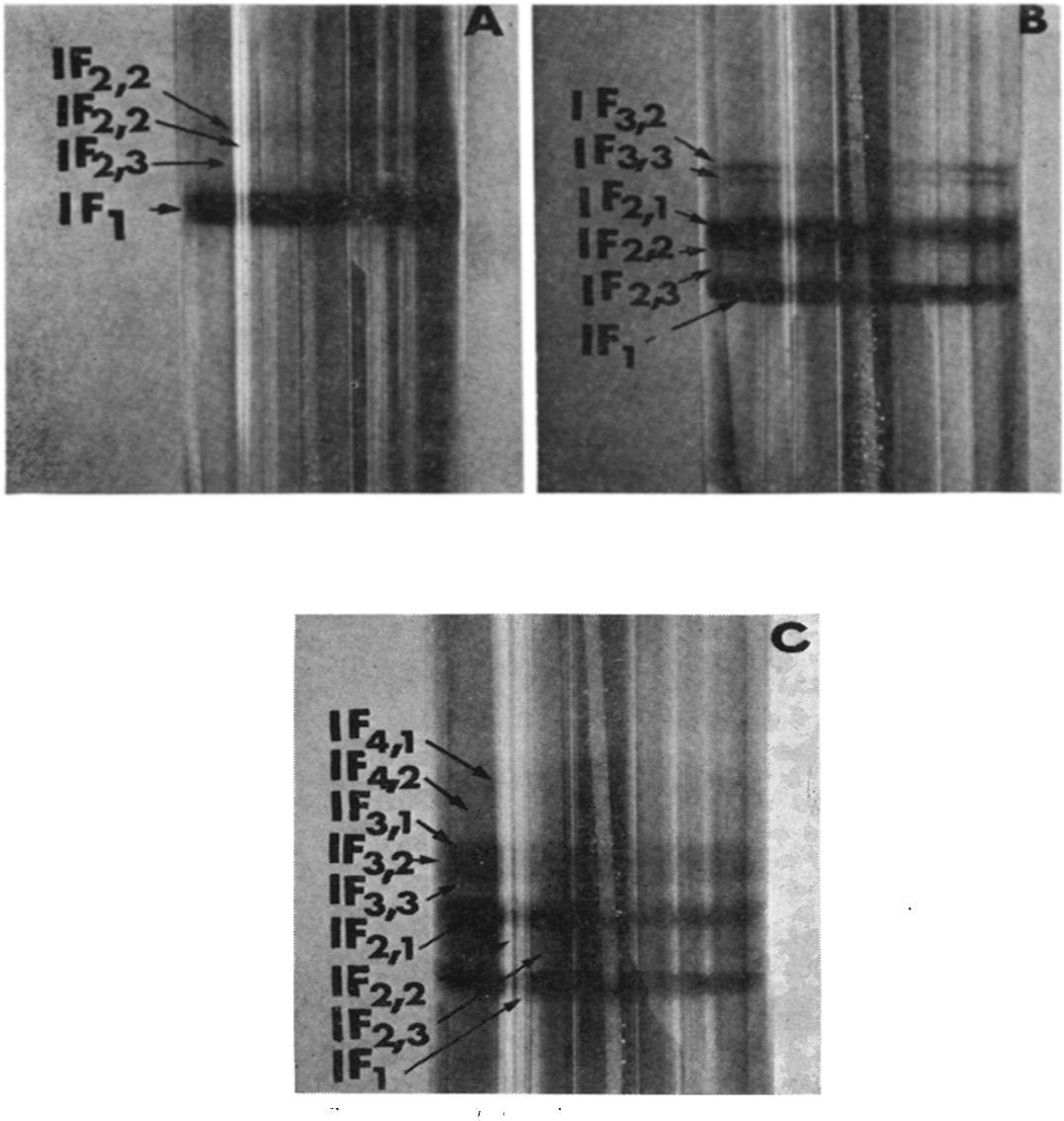

Fig. 3. The MetMb components present in the three fractions from the CM-cellulose chromatography. A shows the MetMb components in fraction I on a pH 6 to 8 isoelectric fractionation column. B shows the MetMb components in fraction II on a pH 5 to 8 column and C shows the components in the fast-moving fraction on a pH 5 to 8 column.

This example illustrates that the isoelectric fractionation apparatus is limited in its ability to detect MetMb components. To be detected, the components must be observed on the column or measured at 409 nm after being eluted from the column.

The amounts of each zone present in each CM-cellulose fraction were determined and are given in Table I. The information given in Table I indicates that the fractions from CM-cellulose column chromatography are very heterogeneous.

TABLE I

COMPOSITION OF EACH METMYOGLOBIN FRACTION FROM CM-CELLULOSE COLUMN CHROMATOGRAPHY

CM-cellulose fraction	*Amount of each zone* (%)			
	IF_1	IF_2	IF_3	IF_4
Fraction I	96.8	3.2		
Fraction II	52.9	40.5	6.6	
Fast-moving fraction	41.8	42.5	13.9	1.8

When a comparison is made between polyacrylamide gel electrophoresis and isoelectric fractionation of the MetMb components, gel electrophoresis was shown to separate four bands from partially purified bovine MetMb, whereas isoelectric fractionation was able to separate four MetMb zones, with each zone having from one to three components. The total number of components obtained from MetMb on the isoelectric fractionation apparatus was nine. We believe that the four zones seen on the isoelectric fraction correspond to the four bands seen on gel electrophoresis. If this is true, the isoelectric fractionation apparatus has shown that each of the gel electrophoretic bands are composed of several components.

Isoelectric fractionation has shown that bovine MetMb consists of nine components. Table II lists each MetMb component and its isoelectric point. The isoelectric point of the first component in each zone differs by approximately 0.03 pH units from the corresponding component in the neighboring zones. In this study we demonstrated that a difference of 0.03 pH units corresponded to a net charge difference of one, by measuring the isoelectric points of the met and oxy forms of the main component. The isoelectric points of these two forms of myoglobin differed by 0.03 pH units. The met and oxy forms are known to differ by one in net charge.

TABLE II

THE ISOELECTRIC POINT OF EACH METMYOGLOBIN COMPONENT AT 23°

Component	*Isoelectric point*	*Average deviation*
IF_1	7.10	±0.03 (6)
$IF_{2,1}$	6.79	±0.03 (6)
$IF_{2,2}$	6.85	±0.02 (5)
$IF_{2,3}$	6.90	±0.01 (4)
$IF_{3,1}$	6.48	±0.01 (2)
$IF_{3,2}$	6.54	±0.01 (2)
$IF_{3,3}$	6.57	±0.01 (2)
$IF_{4,1}$	6.15	—
$IF_{4,2}$	6.35	—

The presence of one unit difference in net charge between a component and its corresponding component in neighboring zones suggests that these components may have arisen from a stepwise cleavage of groups from the MetMb molecule. The stepwise cleavage of amide groups from asparagine and glutamine residues could yield components with larger net negative charges, as are present among the minor MetMb components.

The heterogeneity seen in the MetMb fractions from CM-cellulose column chromatography was thought by ATASSI[8] to have been due to the interconversion of one fraction to form others. In this study, this idea was investigated with the placing of one component, obtained from the isoelectric fraction of bovine MetMb, back on the apparatus and observing the number of MetMb components appearing the second time. The IF_1 component from several runs was collected, dialyzed, lyophilyzed and stored in the freezer for several weeks. This component was then rerun on the isoelectric fractionation apparatus.

After rerunning the IF_1 band, the only MetMb seen on the column was the IF_1 band. A small band just above IF_1 was present and was shown to be oxymyoglobin (MbO_2) by its characteristic spectrum. The appearance of only one MetMb component after a second isoelectric fractionation demonstrated that the major MetMb component was unable to interconvert to form any minor components. The interconversion described earlier may have been due to the heterogeneous nature of the CM-cellulose fractions studied.

The MbO_2 present during the isoelectric fractionation was produced by reduction of MetMb during the experiment. Since all myoglobin placed in the column had been previously oxidized with potassium ferricyanide, any MbO_2 present in the column was formed during the isoelectric fractionation. The appearance of MbO_2 during the isoelectric fractionation may have resulted from the exposure of the MetMb molecule to electrode reaction products that diffuse through the column during the long period of separation (24 to 36 h). Exposure of the MetMb molecule to these products for such a long period of time could cause the reduction of some of the MetMb.

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