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EFFECT OF pH AND COLUMN SIZE ON CM-CELLULOSE CHROMATOGRAPHY OF BOVINE MYOGLOBIN

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SUMMARY

The several fractions obtained by chromatography of bovine myoglobin on CM-cellulose can be explained partially in terms of a pH anomaly. Adsorption of myoglobin on CM-cellulose causes the pH to rise, and unadsorbed myoglobin moves down the column with the solvent front. This fast-moving fraction (FMF) contains the several fractions that make myoglobin microheterogeneous, and the FMF is gradually adsorbed as it moves down the column. Subsequent elution of myoglobin leads to distinct fractions, which are heterogeneous on gel electrophoresis. This explanation of the chromatographic behavior of myoglobin, along with a suggested nomenclature for the chromatographic fractions, should remove some of the confusion concerning the microheterogeneity of myoglobin.

INTRODUCTION

Several people[1–4] have observed the microheterogeneity of myoglobin on CM-cellulose chromatography, but the pattern and number of fractions seem to differ with each observer. Furthermore, the discrete fractions obtained from CM-cellulose columns are frequently heterogeneous upon rechromatography or by gel electrophoresis. While investigating the microheterogeneity, we have made observations of bovine myoglobin on CM-cellulose and have discovered some characteristics of the chromatography that can account for the different patterns and numbers of fractions. In this paper, we give an explanation for these differences and suggest a nomenclature for the fractions, which should help to avoid confusion. The actual cause of the microheterogeneity of myoglobin remains a challenging problem.

MATERIALS AND METHODS

Myoglobin was isolated from bovine leg muscles (semitendinosus, semimembranosus and biceps femoris) and purified by following the procedure of SNYDER AND

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AYRES[5]. Only fractions precipitated after 90% saturation with ammonium sulfate were used. Myoglobin was oxidized to MetMb before adsorption on the columns.

All CM-cellulose used in this study was from the same batch (Sigma Chemical Co.) and was of medium mesh with a capacity of 0.63 mequiv./g. New cellulose (not regenerated) was used for all experiments. Before use, cellulose was suspended in equal volumes of 0.5 *M* NaCl and 0.5 *M* NaOH overnight. It was then washed with deionized water until neutral, and all fines not settling in 15 min were decanted. The final step was equilibration of the cellulose with starting buffers.

Columns were gravity packed. Eluting buffers were composed of 0.01 *M* phosphate and pH gradients for elution were linear. The eluting buffer was pumped through the columns at a rate of 1 ml/min, and 7–8 ml fractions were collected. Absorbance of the fractions was measured at 280 and 409 nm, and the relative amounts of myoglobin were estimated by triangulation of the area under the 280 nm curves.

The numbering of myoglobin fractions eluted from CM-cellulose columns is an important feature of the microheterogeneity problem. A rational method for numbering and referring to the fractions would be most useful in trying to understand and explain the various elution patterns appearing in the literature. We propose that the largest and most positive charged myoglobin component (usually the last component eluted from a CM-cellulose column) be designated fraction I. This fraction is normally so much larger than the others that it is readily identified. We propose that adjacent fractions be numbered II, III, IV, etc., in sequence from fraction I. Usually, the minor components are present in decreasing concentration in the same order as II, III, IV, etc.

There is a myoglobin fraction that can be readily observed but does not fit the numbering system just given. We have named it FMF (fast-moving fraction) for reasons which will be obvious from the results section. Nonheme proteins normally do not present any difficulties or confusion in interpreting elution patterns of myoglobin and do not require a special numbering system.

RESULTS AND DISCUSSION

The frequent separation of MetMb fractions on CM-cellulose columns led us to observe closely what was happening as the colored protein was applied to the column. Invariably, a MetMb fraction moved quickly away from the main MetMb fraction adsorbed at the top of the column. Fig. 1 shows a sequence of photographs depicting this separation of FMF (fast-moving fraction). The FMF decreases in quantity as it moves down the column.

It is possible to elute and collect FMF as a fraction distinct from the main MetMb fraction on a short (5-cm) column. Fig. 2 shows that two distinct fractions are formed on short columns, and the FMF is not due simply to overloading the column. If the column length is extended to 17.5 cm, an elution pattern such as that of Fig. 3 is obtained. The MetMb fractions are numbered according to the system explained in the methods section. Note that the amount of FMF in relation to the other fractions is much less than in Fig. 2. By extending the column length to 60 cm, the FMF is lost entirely as shown in Fig. 4. Although a small colorless protein peak is evident at fraction No. 40, no MetMb peak corresponding to the rate of movement of FMF can be found.

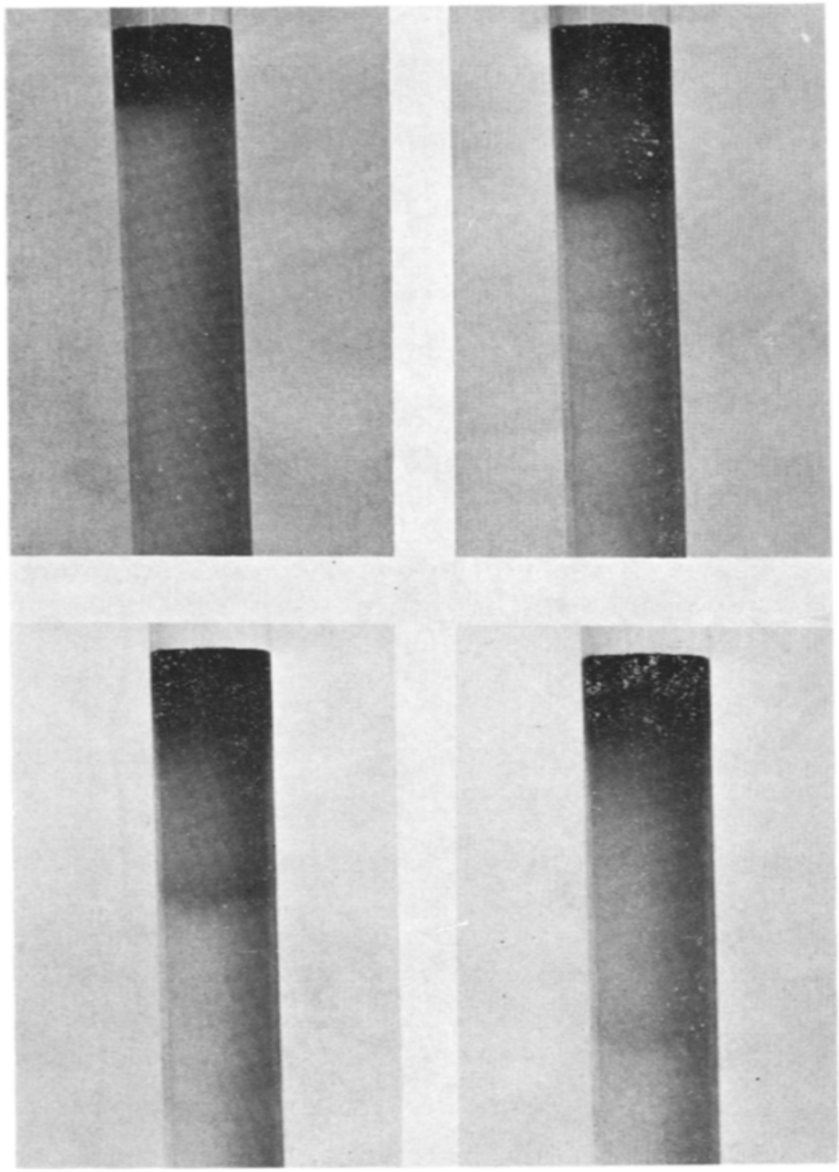
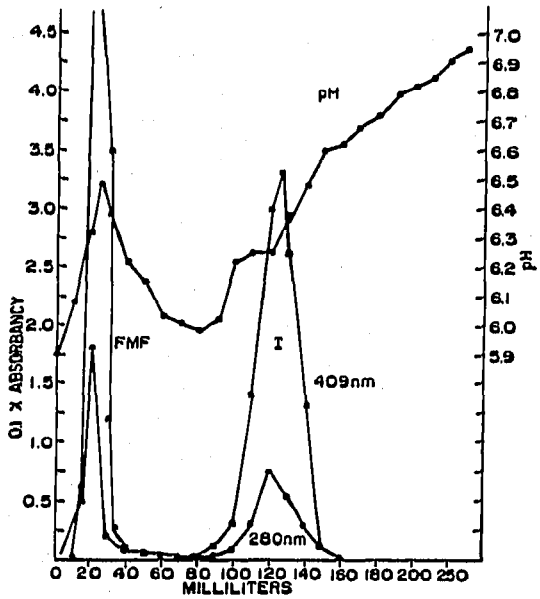


Fig. 1. Formation and re-equilibration of the FMF. Photographs taken at approximately 5 min intervals starting with upper left, then upper right, lower left, and lower right.

Further study of the conditions affecting the size of FMF showed that the volume of the column bed was important in relation to the total amount of MetMb applied to the column. Table I summarizes the results from several columns. If the amount of MetMb per cc of column material is fairly large, as in columns F, G, and J, then the amount of FMF is large. If the amount of MetMb per cc of CM-cellulose is small, as in columns A, B, C, D, and K, then the FMF is small or nonexistent. These results are contrary to the expected behavior of a protein on CM-cellulose. One would not expect to increase the proportion of a separate protein fraction by putting more of the original protein mixture on the column. Nor, would it be expected that the proportion of one of the fractions could be changed by changing the size of the column.



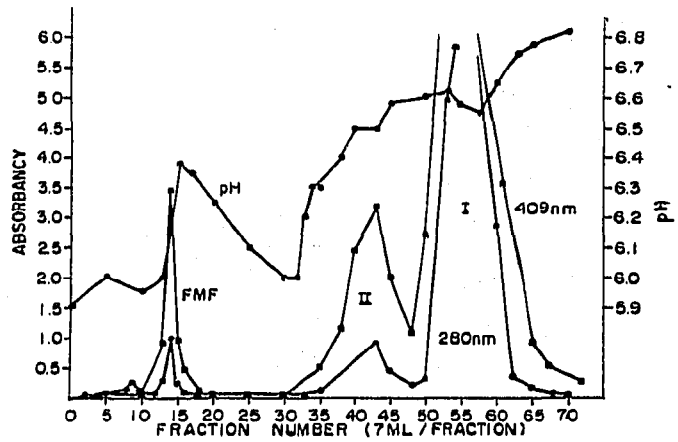


Fig. 2. CM-cellulose chromatogram of bovine metmyoglobin on a 2.5 × 5 cm column. Buffer gradient started at pH 6, and 200 mg of myoglobin was applied.

Fig. 3. CM-cellulose chromatogram of bovine metmyoglobin on a 2.5 × 17.5 cm column. Buffer gradient started at pH 6.0, and 280 mg of myoglobin was applied.

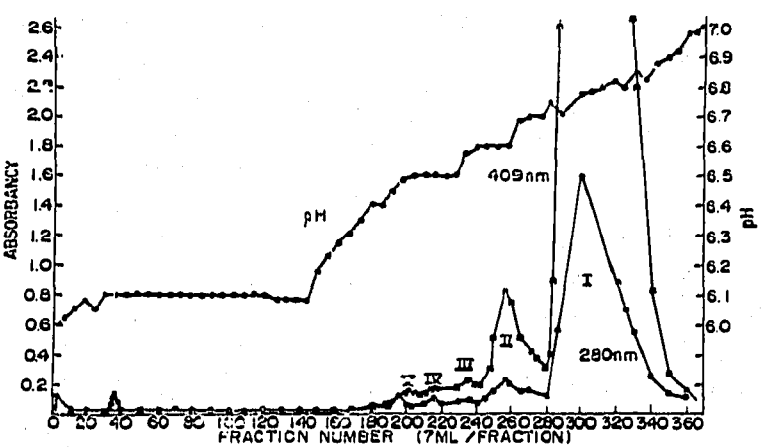


Fig. 4. CM-cellulose chromatogram of bovine metmyoglobin on a 2.5 × 60 cm column. Buffer gradient started at pH 6.0, and 300 mg of myoglobin was applied.

In our study of the relative size of the FMF, we found that it could be influenced by the initial pH of the column. These data are also shown in Table I. If the initial pH was 5.5, even a large proportion of MetMb to CM-cellulose gave a small FMF; columns H and I are examples. In contrast, column E, with a starting pH of 6.8, has a relatively large proportion of FMF even though the amount of MetMb per cc of CM-cellulose is low.

The observation that starting pH had an effect on the relative amounts of FMF caused us to measure pH in the effluent fractions. The results are plotted with

TABLE I

RELATIONSHIP OF EQUILIBRATING pH AND COLUMN CAPACITY TO MYOGLOBIN FRACTIONS ELUTED FROM CM-CELLULOSE COLUMNS

Column designation	*Equilibrating pH*	*Column capacity in cc*	*mg Mb chromatographed*	*mg Mb per cc CM-cellulose*	*Total number fractions eluted*	*Per cent FMF (estimate)*
A	5.9	40.7	125	3.1	3	4%
B	5.9	85.9	280	3.3	3	4%
C	6.0	295	300	1.0	4 or 5	None
D	6.0	43.4	23	0.53	3 or more	None
E	6.8	44.3	22	0.49	2	7%
F	6.0	24.6	250	10.2	2	24%
G	6.0	27.0	206	7.7	2	17%
H	5.5	88.4	437	4.9	4	0.5%
I	5.5	83.5	704	8.4	4	0.3%
J	6.0	39.3	252	6.4	2	8%
K	6.0	39.3	174	4.4	2	2%

the leution patterns in Figs. 2, 3, and 4. We found a sharp increase in pH associated with the FMF, followed by a pH decrease. The increase in pH was most pronounced in very short (5-cm) columns.

Since the columns were being used in a pH region in which the carboxyl groups of the CM-cellulose were not completely ionized, the pH anomaly may have been due to interaction of buffer with CM-cellulose. Hence, we examined the effluent pH from two columns that had no MetMb adsorbed but were treated with a pH gradient. One column was started at pH 5.6, and the second was started at pH 6.0. The pH measurements on effluent fractions are shown in Fig. 5. Both columns showed some buffering activity with an abrupt increase in pH at fraction XXVII for the pH 6.0 column and fraction XXXVI for the pH 5.6 column. These pH changes could not explain the abrupt increase and decrease in pH associated with the FMF. It is surprising that the pH of the column effluent changed as readily as it did. The buffer was only 0.01 *M*, and we know from experience that far more than 600 ml of 0.01 *M* phosphate buffer is required to re-equilibrate a CM-cellulose column from pH 5.5 or 6.0 to pH 7.0. Evidently, only some superficial carboxyls are being titrated, and with sufficient time, the pH of the column effluent would decrease.

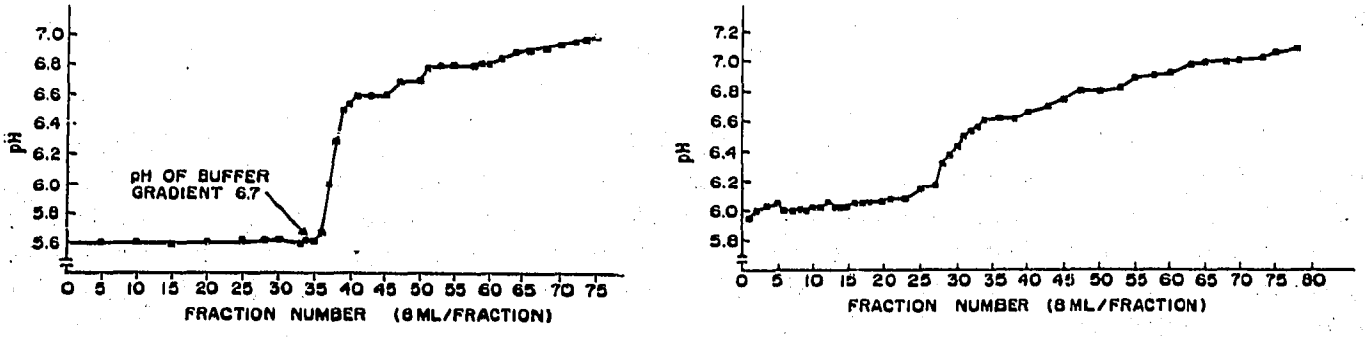


Fig. 5. Titration curves for 2.5 × 18 cm CM-cellulose columns using a linear buffer gradient starting at pH 5.6 for (a) and pH 6.0 for (b). No myoglobin was applied to these columns.

Because of the enhanced pH change with short columns and the failure to find a pH change when myoglobin was not placed on the columns, we concluded that the abrupt pH increase and decrease was probably associated with the initial adsorption of MetMb to the CM-cellulose. CLAYTON AND BUSHUK[6] predicted that such a pH change should take place if the eluting solvent were insufficiently buffered and if the cations replaced by the protein were more basic than the cation groups on the protein.

Such an explanation provides some understanding of the origin of the FMF and of the relationship between the proportion of MetMb per cc of CM-cellulose and the size of the FMF. If the pH increases abruptly as MetMb is bound to CM-cellulose, then MetMb in buffer at pH 6.3 or 6.4 will not be adsorbed and will begin to move down the column. The more MetMb applied to the column originally, the larger will be the MetMb fraction (which would be the FMF) moving down the column. For a short column, the FMF is eluted in the presence of the buffer with higher pH. If the column is long, there is increased opportunity for the buffer to interact with CM-cellulose and gradually decrease in pH. As the buffer decreases in pH, the MetMb being carried by the buffer will begin to interact with the CM-cellulose and be slowly adsorbed as it moves down the column. With a long column, the FMF will be completely adsorbed, and the initial pH increase will not be evident as in Fig. 4.

This explanation of the FMF is helpful in understanding another anomaly of separating MetMb fractions on CM-cellulose. Except for fraction I, the well-defined MetMb fractions that can be eluted from CM-cellulose are far from pure as analyzed by gel electrophoresis. Although fraction I is more pure than the MetMb before chromatography, fraction II and FMF are still heterogeneous. Attempts to minimize the heterogeneity by extruding the columns before elution of fractions and by cutting the columns in sections, from which fractions could be eluted, were not successful in decreasing the heterogeneity.

If the increased pH is responsible for the FMF and if the FMF is gradually readsorbed as it moves down the column, then the origin of the heterogeneity in all but fraction I is not difficult to understand. With MetMb spread over a large section of the column, it would be extremely difficult to get elution of homogeneous MetMb fractions. That distinct (but heterogeneous) MetMb fractions are eluted from CM-cellulose columns may be due to a poor pH gradient. Even though a linear pH gradient was attempt, the pH measurements in Figs. 2, 3, and 4 show some distinct pH steps, with an actual decrease during elution of fraction I. The decrease in pH during elution of fraction I is presumably due to the reverse of the process that causes the rise in pH when fraction I was adsorbed. The origin of the other discontinuities in pH is not known, but abrupt pH changes could cause the appearance of artifactual fractions. An abrupt change in pH after elution of the main MetMb fraction I is sufficient to cause the appearance of a new MetMb fraction following I, but with a pH gradient, no fractions have been observed following fraction I.

With MetMb spread over the column, any stepped elution of the column would be certain to elute MetMb fractions that would be heterogeneous upon rechromatography. The 11 fractions of sperm whale MetMb found by ATASSI[1] may be in this category. ATASSI[1] interpreted the heterogeneity upon rechromatography as evidence for an interconversion of the fractions, but the same result would be obtained if the original fractions were heterogeneous.

We are uncertain about the applicability of these results to other proteins, but,

in any chromatography experiment in which discrete but heterogeneous fractions are obtained from CM-cellulose, the possibility of a pH change in the buffer causing the anomalous results should be explored.

Our results do not help to explain the actual reasons for microheterogeneity in purified myoglobin samples. The fractions obtained from CM-cellulose, however, have been difficult to understand in relation to the fractions obtained by gel electrophoresis. Our results are helpful in understanding why the CM-cellulose fractions are heterogeneous on gel electrophoresis.

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