The Chromatography of the Meromyosins on Diethylaminoethylcellulose

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In a previous chromatographic study of rabbit L-myosin (Perry, 1960) the distribution of adenosine-triphosphatase activity in the main eluted fraction of this protein did not appear to be compatible with enzymic homogeneity. Brahm (1959, 1960) has also reported the separation of components of different adenosine-triphosphatase activity from rabbit myosin. The relation of these findings to the subunit structure of myosin is far from clear but they suggested that re-investigation of the distribution of adenosine-triphosphatase activity between the light and heavy meromyosins (Szent-Gyorgyi, 1953; Gergely, 1953; Mihalyi & Szent-Gyorgyi, 1953) might throw some light on this problem. There is accumulating evidence of the heterogeneity of the meromyosins (Lowe & Holtzer, 1959; Fryar & Gibbs, 1960; Szent-Gyorgyi, Cohen & Philpott, 1960) and the solubility properties of heavy meromyosin compared to myosin and to light meromyosin make this protein particularly suitable for chromatographic studies with diethylaminoethylcellulose.

The present paper is concerned with a chromatographic investigation of the standard light and heavy meromyosin preparations and the distribution of adenosine-triphosphatase activity in the fractions which can be isolated from these proteins. Evidence is presented which suggests that fragments, other than heavy meromyosin, which result from tryptic digestion of myosin, possess appreciable adenosine-triphosphatase activity. A preliminary note of some of the findings reported here has appeared earlier (Mueller & Perry, 1960).

METHODS

Chromatography. Diethylaminoethylcellulose was prepared by the method of Peterson & Sober (1956) and the conditions of chromatography were as previously described (Perry, 1960). Commercial diethylaminoethylcellulose (Whatman powder DE 50), manufactured by W. and R. Balston Ltd., was not found satisfactory for separating fractions from heavy meromyosin. Protein solutions were equilibrated against the appropriate buffer by dialysis and

centrifuged for 20 min. at 10 000g before application to the column, which had previously been fully equilibrated against the buffer. Usually 150–300 mg. of protein was applied in 1.0–1.5% solution to a 2 cm. $\times$ 20 cm. column containing about 7 g. of diethylaminoethylcellulose. Elution was carried out at 60 cm. water pressure and a flow rate of 5–10 ml./hr. For gradient elution a 250 ml. conical mixing chamber was used. Elution was followed by measurement of $E_{1\text{cm}}$ at 280 and 260 m μ . Estimations of protein recovery were made by measuring the areas of the eluted peaks on the plotted chromatograms. All chromatographic procedures and preparations of protein solutions were carried out at 1–2°.

Heavy meromyosin. L-Myosin, prepared as described by Perry (1955), was digested with trypsin at 23° for 10 min. by the method of Szent-Gyorgyi (1953) and immediately dialysed against 10 vol. of 6.7 mM-phosphate Sørensen buffer (pH 7.0). The precipitated light meromyosin was separated by centrifuging; the heavy meromyosin was salted out by $(\text{NH}_4)_2\text{SO}_4$ in the range 40–55% saturation, dissolved in 0.15 M-KCl–20 mM-2-amino-2-hydroxymethylpropane-1:3-diol(tris)-HCl (pH 7.6) and stored at 1–2°.

Light meromyosin. The digestion, carried out as described for heavy meromyosin, was usually stopped after 12 min. and the digest kept at 1–2° for 10 days (Szent-Gyorgyi, 1953). The light meromyosin was precipitated by dialysis against 10 vol. of 6.7 mM-phosphate Sørensen buffer (pH 7.0), the precipitate washed two or three times with the same buffer and redissolved by addition of solid KCl to bring the concentration to 0.5 M. This process was repeated twice more and the light meromyosin stored in 0.5 M-KCl.

Enzyme assays. Adenosine-triphosphatase assays were carried out at 25° and pH 7.6 as described by Perry (1960). Three different concentrations of enzyme from a given fraction were used and specific activities were calculated from the straight-line graphs obtained by plotting phosphate liberated against amount of protein. Specific activities were expressed as $\mu\text{g.}$ of phosphorus liberated in 5 min. by 1 ml. of protein solution of $E_{1\text{cm}}$, 1.0 at 280 m μ ; 1 ml. of protein solution of $E_{1\text{cm}}$ at 280 m μ contained, for L-myosin, light meromyosin and heavy meromyosin, 0.272, 0.320 and 0.248 mg. of N respectively.

Measurements of pH. Buffers were diluted to the concentration used and their pH was measured at room temperature (about 18°) with the glass electrode (Electronic Instruments Ltd.). The electrode was standardized at pH 6.99 with Soloid standard buffer tablets (Burroughs Wellcome and Co.).

Materials. The trypsin used was a twice-recrystallized preparation containing 50% of MgSO_4 (supplied by Sigma Chemical Co.). The soya-bean trypsin inhibitor was

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Ultracentrifuging. Sedimentation studies were carried out at 20° on the Spinco analytical ultracentrifuge, model E. The composition of the solvent was 0.15M-KCl-20 mM-tris-HCl (pH 7.6) in all experiments.

Starch-gel electrophoresis. The procedure was carried out in 22 mM-sodium borate buffer (pH 9.1) according to Smithies (1955), with the modification of Hall (1959). The protein was applied on filter-paper strips, the operating potential was 4-6 v/cm. and electrophoresis was carried out for 3-6 hr. at room temperature (about 18°).

RESULTS

Heavy meromyosin

Application of the heavy meromyosin preparations purified with $(\text{NH}_4)_2\text{SO}_4$ to diethylaminoethylcellulose columns equilibrated against 0.15M-KCl-20 mM-tris-HCl (pH 7.6 or 8.3) yielded two main fractions (Fig. 1). Under these conditions 11-17% of the total material applied, estimated on an E_{280} basis, was not held on the column and passed through unretarded (fraction H1). The bulk of the material (fraction H2) was eluted with a peak at 0.20M-KCl-20 mM-tris-HCl (pH 7.6 or 8.3) on application of a gradient to the column. This fraction represented 83-89% of the total

Fig. 1. Chromatography of heavy meromyosin on diethylaminoethylcellulose. Protein solution (25 ml.; $E_{1\text{cm}}$ 7.1 at 280 $m\mu$) in 0.15M-KCl-20 mM-tris-HCl (pH 8.3) was applied to a column 21.5 cm. $\times$ 2 cm. diam. Gradient to 0.35M-KCl-20 mM-tris-HCl at point marked A and step to 2.0M-KCl-20 mM-tris-HCl at B. O, E at 280 $m\mu$; ●, specific adenosine-triphosphatase (ATPase) activity; ■, chloride concentration.

material eluted. The peak with a maximum at a chloride concentration of 0.195-0.205 equiv./l. was fairly symmetrical, although a moderate degree of trailing was apparent with gradient elution. Stepwise application of 0.21M-KCl-20 mM-tris-HCl (pH 7.6) eluted fraction H2 as a sharp peak. Subsequent application of 2M-KCl-20 mM-tris-HCl (pH 7.6) eluted an insignificant amount of material absorbing at 280 or 260 $m\mu$ (less than 1% of total eluted material).

Re-chromatography of fraction H2 obtained from the original purified heavy meromyosin preparation by stepwise elution between 0.15M-KCl and 0.35M-KCl in 20 mM-tris-HCl (pH 7.6) indicated that a practically complete separation from fraction H1 was obtained (Fig. 2).

Adenosine-triphosphatase activity. Study of the specific adenosine-triphosphatase activity of the fractions obtained by chromatographing purified heavy meromyosin revealed that significant activity was associated with both peaks (Fig. 1). The specific activity remained constant throughout the major portion of the H2 peak and although some fall could be detected in the trailing edge, the picture in general was of enzymic homogeneity throughout the bulk of the fraction. The average specific activity of fraction H2 was 240 units. This represented about a 20% increase on the unchromatographed heavy meromyosin preparations, which were themselves about 50% more active than the original L-myosin preparations from which they were obtained.

Fraction H1 was much less enzymically homogeneous. The earliest eluted fractions approached the original heavy meromyosin in specific activity but the enzymic activity fell off sharply in a linear fashion in subsequent fractions so that at the E_{280} peak the activity had fallen by more than 50% (Fig. 1). The average specific activity, determined on the combined fractions of peak H1, was equal to about 40% of that of fraction H2. The high values of the faster components of the H1 peak varied somewhat, but never reached the values obtained for specific adenosine-triphosphatase activity of the H2 peak.

The enzymic activity of fraction H1 did not appear to be due to gross contamination with material from fraction H2, for its chromatographic characteristics were different and on re-chromatography the fraction H1 was virtually completely eluted when the buffer concentration reached 0.15M-KCl-20 mM-tris-HCl (pH 7.6) (Fig. 2).

Ultracentrifuge studies. Ammonium sulphate-purified heavy meromyosin sedimented in the ultracentrifuge as a sharp peak. If concentrations of the protein were sufficiently high the presence of slower components could be detected by (a) the blunting of the angle of the trailing edge, (b) slight

Fig. 2. Re-chromatography of fractions isolated from heavy meromyosin. (i) Heavy meromyosin. Protein solution (50 ml.; $E_{1\text{ cm.}}$ 3.0 at 280 $m\mu$) in 0.15 M-KCl-20 mm-tris-HCl (pH 7.6) was applied to a column 19.5 cm. $\times$ 2 cm. diam. Step to 0.35 M-KCl-20 mm-tris-HCl at A. Portions of peaks between broken lines were used for re-chromatography. (ii) Re-chromatography of fraction H1. Protein solution (64 ml.; $E_{1\text{ cm.}}$ 0.198 at 280 $m\mu$) in 20 mm-tris-HCl (pH 7.6) was applied to a column 7.0 cm. $\times$ 1.0 cm. diam. Step to 0.15 M-KCl-20 mm-tris-HCl at B and step to 0.35 M-KCl-20 mm-tris-HCl at C. (iii) Re-chromatography of fraction H2. Protein solution (44 ml.; $E_{1\text{ cm.}}$ 2.16 at 280 $m\mu$) in 0.15 M-KCl-20 mm-tris-HCl (pH 7.6) was applied to a column 19.0 cm. $\times$ 2 cm. diam. Step to 0.35 M-KCl-20 mm-tris-HCl at D.

elevation of the base line on the meniscus side and (c) the appearance of a small slow peak (Fig. 3a). The exact pattern varied somewhat in different preparations. In aged preparations faster peaks, presumably due to aggregation, appeared.

Chromatography on diethylaminoethylcellulose removed the slower components, for even at high concentrations fraction H2 sedimented as a single peak (Fig. 3b). The base line from the peak to the meniscus was essentially straight and there was no blunting of the angle the peak made with the base line. In fraction H1, concentrated by vacuum dialysis or by precipitation with 90% $(\text{NH}_4)_2\text{SO}_4$, at least three components could be recognized on ultracentrifuging (Fig. 3c).

Confirmation of the heterogeneity of fraction H1 was obtained by starch-gel electrophoresis. This fraction moved as three well-defined bands and occasionally a fourth could be recognized. Fraction H2 showed only slight irregular movement and gave no defined pattern under our conditions of electrophoresis.

Ammonium sulphate fractionation. In precipitating sharply between 45 and 50% of $(\text{NH}_4)_2\text{SO}_4$, fraction H2 resembled unchromatographed heavy

meromyosin (Fig. 4). The precipitation of the H1 fraction, however, in general extended over a broader zone of $(\text{NH}_4)_2\text{SO}_4$ concentrations. Even at 80% saturation precipitation was not complete (Fig. 4).

Light meromyosin

In the region of I 0.15 light meromyosin is insoluble at pH 7.6 but satisfactory solutions for chromatography were obtained by increasing the pH to 8.2. In this respect light meromyosin resembled L-myosin (Perry, 1960). When applied in 0.15 M-KCl-20 mm-tris-HCl (pH 8.25) to a column of diethylaminoethylcellulose previously equilibrated against the buffer, light meromyosin was separated into three distinct fractions (Fig. 5). Fraction L1 passed through the column with the original eluent without apparent retardation and was eluted as a fairly symmetrical peak. Further elution of protein began when the chloride concentration of the buffer was gradually raised by a gradient at 0.185–0.195 equiv./l. The elution of this fraction (fraction L2) continued over a wide range of rising KCl concentration, leading to a rather broad asymmetric and trailing peak. The maximum occurred at 0.20–0.21 equiv. of chloride/l. but in

Fig. 3. Ultracentrifuge diagrams of heavy meromyosin and the fractions obtained from it by chromatography on diethylaminoethylcellulose. Solvent: 0.15M-KCl-20 mM-tris-HCl (pH 7.6). Speed: 59 780 rev./min. Temp.: 20°. (A) Heavy meromyosin purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation between 40 and 55% saturation: (i) 19 min., 65°; (ii) 35 min., 65°; (iii) 59 min., 65°; (iv) 75 min., 35°; (v) 91 min., 35°. (B) Heavy meromyosin fraction H2. Eluted after removal of the H1 fraction by stepwise raising of the salt concentration from 0.15M-KCl- to 0.35M-KCl-20 mM-tris-HCl (pH 7.6): (i) 18 min., 65°; (ii) 34 min., 65°; (iii) 58 min., 65°; (iv) 74 min., 35°; (v) 90 min., 35°. (C) Heavy meromyosin fraction H1. This fraction passed unheld through the diethylaminoethylcellulose column when heavy meromyosin was applied in 0.15M-KCl-20 mM-tris-HCl (pH 7.6). (i) 9 min., 65°; (ii) 25 min., 65°; (iii) 49 min., 35°; (iv) 73 min., 35°; (v) 97 min., 35°.

Fig. 4. Solubility of fractions H1 and H2 from heavy meromyosin in $(\text{NH}_4)_2\text{SO}_4$ solutions. Solid $(\text{NH}_4)_2\text{SO}_4$ added to about 4 mg. of protein in a final volume of 5 ml. Protein precipitated was estimated by measuring E_{280} of the supernatant after high-speed centrifuging. ○, Fraction H1; ●, fraction H2.

general the peak was much broader than that of the H2 fraction from heavy meromyosin. Re-chromatography of fraction L2 indicated that its behaviour was consistent and that the initial separation effectively removed fractions L1 and L3.

Application of 2M-KCl-20 mM-tris-HCl (pH 8.2) after fraction L2 had been completely removed from the column eluted a third fraction (fraction L3). This fraction differed from the two preceding fractions in that E_{260} in the ascending portion of the peak rose relative to E_{280} and exceeded it over most of the peak. Fraction L3 was probably similar to, or derived from, the ribonucleoprotein component previously isolated from myosin by chromatography (Perry, 1960). From the ratios of E at 260 and 280 $m\mu$ (Warburg & Christian, 1941) the nucleic acid content of this fraction was in the range 10–20%.

Fractions L1 and L2 were fairly constant in amount, representing on an E_{280} basis 12–15 % and 70–80 % respectively of the eluted protein. Fraction L3 showed the greatest variation, ranging from 6 to 25 %. This was no doubt due to the fact that because of the appreciable trailing of fraction L2 it was sometimes impracticable to wait until the eluate had reached an E_{280} of zero before applying the step to 2M-KCl–20 mM-tris-HCl (pH 8.2).

Adenosine-triphosphatase activity. Unchromatographed light meromyosin possessed 15–20 % of the specific adenosine-triphosphatase activity of the original L-myosin. This activity could not be removed either by repeated precipitation of the light meromyosin or by increasing the time of the tryptic digestion of the L-myosin from 10 to 12 min.

The distribution of enzymic activity in the fractions separated from light meromyosin is shown in the upper part of Fig. 5, in which the specific adenosine-triphosphatase activity is plotted against eluate volume. Clearly the major portion of the activity was associated with fraction L2, where it showed a characteristic and consistently observed distribution. The specific activity was highest at the front of the peak, rapidly fell in the next few fractions and then remained fairly constant at a level slightly below that of the unchromatographed preparation. In spite of this consistent pattern of distribution, in different preparations the actual values ranged between 100 and 200 units at the front and between 50 and 15 units for the remaining portion of the peak. A barely significant level of adenosine-triphos-

phatase activity, which was too low to establish a distribution pattern, was observed in fraction L1. Enzymic activity was consistently present in fraction L3, although the activity was lower than that in fraction L2. The pattern of distribution was consistent but the adenosine triphosphatase tended to be higher the higher the proportion of the total eluted material represented by fraction L3. Highest values were obtained in the front of the L3 peak and the activity declined in a fairly linear fashion to the trailing edge.

DISCUSSION

Although the standard preparations of both light and heavy meromyosin are heterogeneous by chromatographic criteria the major fraction obtained from both proteins behaved on diethyl-aminoethylcellulose very much like the parent myosin molecule (Perry, 1960), i.e. in each case the major eluted peak is obtained at about I 0.2 in the range pH 7.6–8.2.

As was originally reported by Szent-Gyorgyi (1953), we find relatively low but significant adenosine-triphosphatase activity in the light meromyosin fraction. This activity is largely confined to the principal fraction, L2, which itself appears to be enzymically heterogeneous in so far as the specific activity varies in a marked and characteristic way along the eluted peak. Although any undigested myosin would be expected to be found in this fraction the evidence available suggests that myosin is not responsible for the enzymic activity. The evidence available suggests that it

Fig. 5. Chromatography of light meromyosin. Protein solution (17 ml.; $E_{1\text{ cm.}}$ 4.5 at 280 $m\mu$) in 0.15M-KCl–20 mM-tris-HCl (pH 8.3) applied to a column 10.5 cm. $\times$ 2 cm. diam. Gradient to 0.35M-KCl–20 mM-tris-HCl was applied at point A and step to 2.0M-KCl–20 mM-tris-HCl at B. $\circ$, E at 280 $m\mu$; $\bullet$, specific adenosine-triphosphatase (ATPase) activity; $\blacksquare$, chloride concentration.

may be an oversimplification to assume that light meromyosin is devoid of enzymic activity.

The ribonucleoprotein normally associated with myosin preparations (Mihalyi, Laki & Knoller, 1957; Liubimova & Fain, 1958; Perry & Zydowo, 1959; Perry 1960) is entirely associated with the light meromyosin fragment. Szent-Gyorgyi *et al.* (1960) have recently independently reported a similar association. If this ribonucleoprotein has any biosynthetic function (cf. Perry & Zydowo, 1959) its presence may explain the reported differences in the turnover of light and heavy meromyosins (Velick, 1956; Schapira, Broun, Dreyfus & Kruh, 1956).

On the other hand, the main component fraction H2 isolated from heavy meromyosin by the chromatographic technique presents convincing evidence of a high degree of homogeneity from sedimentation studies and from its behaviour on re-chromatography. Further, the specific adenosine-triphosphatase activity of this component was consistently about 20 % higher than that of unchromatographed meromyosin, and a high degree of enzymic homogeneity was indicated by the fact that specific activity remained remarkably constant over most of the eluted peak. A fall in activity in the last few fractions could be due to inactivation of the enzyme, but if the decrease was the consequence of the presence of a less readily eluted contaminant the amount present could not have exceeded a low percentage of the whole. The distribution of adenosine-triphosphatase activity over the eluted peak of fraction H2 is much more compatible with the concept of a homogeneous enzyme preparation than are the results with myosin. The results obtained with the subunit emphasize the significance of those obtained with myosin but as yet do not indicate an explanation of the earlier findings (Perry, 1960; Brahms, 1959, 1960).

The fraction not held by diethylaminoethyl-cellulose at 0.15M-potassium chloride-20 mM-tris-hydrochloride (pH 7.6 or 8.2), usually representing 10-15 % of the heavy meromyosin preparations, is of particular interest. It contains components which are probably responsible for the heterogeneity observed in ultracentrifuge studies by other workers (Lowey & Holtzer, 1959; Fryar & Gibbs, 1960; Mihalyi & Harrington, 1959), and might on general grounds be presumed to be of smaller molecular size than heavy meromyosin itself. This fraction possesses appreciable adenosine-triphosphatase activity, and although the possibility of slight H2 contamination cannot be completely excluded it is unlikely that it accounts for the enzymic activity of this fraction. In view of the sedimentation results, the consistent chromatographic behaviour, the salting-out characteristics

and electrophoretic behaviour the evidence is strongly suggestive that there exist in the standard heavy meromyosin preparations protein fragments, other than the main component, which possess adenosine-triphosphatase activity. This conclusion is supported by the results of preliminary experiments which indicate that the H2 component can be degraded still further with trypsin to produce fragments similar to those present in the H1 fraction.

The marked heterogeneity apparent in both light and heavy meromyosin preparations emphasizes the need for caution in interpreting the results of a number of important studies on these components, i.e. end-group analysis (Middlebrook, 1958, 1959), turnover studies (Velick, 1956; Schapira *et al.* 1956) and investigations based on antigenic properties (Marshall, Holtzer, Finck & Pepe, 1959; Szent-Gyorgyi & Holtzer, 1960).

SUMMARY

1. About 85 % of the standard preparations of heavy meromyosin can be shown by chromatography on diethylaminoethylcellulose to consist of a component, fraction H2, which is of high homogeneity by sedimentation, chromatographic and enzymic criteria. The enzyme activity of this fraction was higher than that of the unchromatographed heavy meromyosin and the myosin from which it was derived.

2. The minor fraction separated from heavy meromyosin, fraction H1 has been shown to consist of at least three components by sedimentation studies and starch-gel electrophoresis. Evidence of enzymic heterogeneity in this fraction is also presented.

3. Appreciable adenosine-triphosphatase activity is associated with the H1 fraction and the results strongly suggest that it contains fragments smaller than the H2 component derived either from the latter or from the myosin molecule during the trypsin treatment.

4. Light meromyosin has been separated into three main fractions, one of which is rich in nucleic acid.

5. Small but significant amounts of adenosine-triphosphatase activity were present in all preparations of light meromyosin.

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