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The Biological Activity of Subfragment 1 Prepared from Heavy Meromyosin

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1. The action of trypsin, chymotrypsin and subtilisin on the adenosine-triphosphatase and actin-combining activities, as measured by viscometric means, of H-meromyosin were compared. 2. Subfragment 1 produced by prolonged tryptic digestion has a molecular weight of 129000. 3. The preparations isolated by gel filtration and actin combination were shown to be similar. 4. Subfragment-1 preparations possess appreciably higher adenosine-triphosphatase activities than H-meromyosin when related to total nitrogen. 5. Chromatographic and gel-filtration studies indicated that adenosine-triphosphatase activity is not distributed uniformly in all fractions of subfragment 1. 6. The Ca^{2+}-activated adenosine triphosphatase of subfragment 1 was stimulated by thiol reagents in a similar fashion to myosin and H-meromyosin. 7. Subfragment 1 differed from myosin and H-meromyosin in that its adenosine triphosphatase was only slightly activated by Mg^{2+} in the presence of actin. 8. A subfragment-1-like component was obtained by chymotryptic digestion of H-meromyosin. 9. The results obtained from enzymic and hydrodynamic studies and from amino acid analyses are compatible with the concept of one molecule of H-meromyosin giving rise to one molecule of subfragment 1 on proteolytic digestion.

In previous studies from this Laboratory (Mueller & Perry, 1961, 1962) it was shown that H-meromyosin prepared by tryptic digestion of myosin, could be further degraded to a considerably smaller fragment, subfragment 1, which retained the actin-combining and ATPase* properties of the original myosin molecule. The physical properties and appearance in the electron microscope (Rice, 1961, 1964) of subfragment 1 indicate that it is a relatively globular particle, and values for the molecular weight have ranged from 100000 to 170000 (Mueller & Rice, 1964; Young, Himmelfarb & Harrington, 1964; Mueller, 1965), the precise value probably depending on the extent of digestion taking place during preparation. On balance the evidence available suggests that subfragment 1 is derived from the thickened end of the myosin molecule (Cohen, 1961; Holtzer, Lowey & Schuster, 1962; Lowey & Cohen, 1962), and, assuming a one-to-one relationship, represents about one-quarter to one-fifth of the original myosin molecule. However, Young, Himmelfarb & Harrington (1964, 1965), largely from the analysis of sedimentation studies of short tryptic digests of H-meromyosin, have postulated that subfragment 1 arises from the dissociation of one molecule of partly digested H-meromyosin into three similar sub-units of molecular weight in the range 100000–121000. Each of these fragments is considered to possess an active site and to represent a part of one of the original three polypeptide chains that have previously been postulated (Kielley & Harrington, 1960; Woods, Himmelfarb & Harrington, 1963) as the sub-units of myosin. According to this hypothesis subfragment 1 would represent at least 60% of the original myosin molecule. On the other hand, Mueller (1965) implies that subfragment 1 accounts for 38–41% of the myosin molecule.

In view of the evidence for the association of actin-combining and ATPase activities with different sites on the myosin molecule (Perry & Cotterill, 1965*b*), it is possible that fragments with which these properties are separately associated might be isolated after prolonged digestion of H-meromyosin. One of the aims of this investigation was to investigate this possibility, and since the work described in this paper was started a report has appeared claiming the isolation of an ATPase-active fragment that does not combine with actin (Nankina, Kofman, Chernyak & Kalamkarova, 1964). The present study has been particularly concerned with the study of the biological activity of subfragment 1 prepared by prolonged digestion of H-meromyosin, and the relation of this fragment to the structure of the original myosin molecule.

* Abbreviation: ATPase, adenosine triphosphatase.

METHODS

Preparation of muscle proteins. L-myosin prepared by the method of Perry (1955) was converted into H-meromyosin by digestion with crystalline trypsin (0·045 mg./ml.) by a method otherwise similar to that of Szent-Gyorgyi (1953). Muscle acetone-dried fibre prepared by the method of Barany, Biro, Molnar & Straub (1954) was used for the preparation of G-actin, which was extracted by the method of Straub (1943) for 45 min. at 3° to decrease tropomyosin contamination (Drabikowski & Gergely, 1962). Purification and conversion into F-actin was carried out by isoelectric precipitation with 10 mM-sodium acetate buffer, pH 4·7.

Preparation of subfragment 1. H-meromyosin (approx. 5 mg./ml.) in 25 mM-tris–HCl buffer, pH 7·6, was brought to 25° in a water bath and 0·1 vol. of trypsin solution (2·5 mg./ml. in mN-HCl), prepared 30–60 min. before use, was added. After 90 min. the digestion was stopped with 0·2 vol. of soya-bean trypsin inhibitor (2·5 mg./ml.) in 25 mM-tris–HCl buffer, pH 7·6, and subfragment 1 isolated from the digest by either of the following procedures.

(*a*) Gel filtration. The precipitate obtained by adding 35·1 g. of $(NH_4)_2SO_4$/100 ml. of digest was separated by centrifugation at 34000*g* for 15 min., dissolved in 3–4 ml. of water and dialysed for 18 hr. against several changes of 25 mM-tris–HCl buffer, pH 7·6. After centrifugation at 100000*g* for 1 hr. the solution was applied to a Sephadex G-200 column that had been equilibrated with 25 mM-tris–HCl buffer, pH 7·6, for at least 24 hr. Protein was eluted at 10–15 ml./hr. in 3–3·5 ml. fractions and protein concentration followed by the measurement of $E_{280}^{1cm.}$. The fractions representing the main portion of the second peak eluted were combined and in some cases re-run on Sephadex G-200 for further purification.

(*b*) Actin combination. To every 100 ml. of digest, 10 ml. of F-actin (6–7 mg./ml.) in 10 mM-$MgCl_2$–25 mM-tris–HCl buffer, pH 7·6, was added. The 'acto-digest' solution was centrifuged at 80000*g* for 3 hr. and the opalescent pellet of complex and any uncombined actin dispersed in 10 ml. of dissociation buffer consisting of 0·5 M-KCl–mM-$MgCl_2$–5 mM-sodium pyrophosphate–25 mM-tris–HCl buffer, pH 7·6, in a hand homogenizer. This solution was centrifuged for 3 hr. at 100000*g* and the supernatant dialysed overnight against 4 l. of 25 mM-tris–HCl buffer, pH 7·6, to remove pyrophosphate. After further centrifugation for 1 hr. at 100000*g*, the preparation was stored at 0° at approx. 8 mg./ml.

Chromatography. Unless otherwise stated DEAE-cellulose was prepared by the method of Peterson & Sober (1956) and chromatography was carried out under the general conditions described by Perry (1960).

Enzymic studies. These were in general carried out in 2 ml. at 25° as described by Perry (1960). For experiments with thiol inhibitors, the enzyme was usually treated with the inhibitor for 10 min. at 0° in the incubation systems indicated in the text less ATP, brought to 25° and the reaction started by the addition of ATP. All specific activities, unless otherwise stated, were determined in 5 mM-$CaCl_2$–5 mM-ATP–57·5 mM-tris–HCl buffer, pH 7·6, and expressed as phosphate (in μg. of P) liberated by 1 ml. of enzyme solution with $E_{280}^{1cm.}$ 1·0 in 5 min. under these conditions.

Sedimentation and diffusion measurements. Sedimentation coefficients were evalued by the procedure of Schachman (1957) by using the data obtained with the Spinco model E analytical ultracentrifuge. All runs were carried out at 20° in 0·15 M-KCl–20 mM-tris–HCl buffer, pH 7·6, and corrections to relate the results to water were made with the standard equation (Schachman, 1957). Diffusion measurements were made at 20° with the double-sector synthetic-boundary cell and the diffusion coefficients determined and corrected to water, also by the procedure of Schachman (1957).

Viscosity measurements. These were carried out at 0° with Ostwald viscometers of capacity 3 ml. and out-flow time for 25 mM-tris–HCl buffer, pH 7·6, of 38–60 sec. Actin combination was followed in digests as follows. To 4 ml. of digest, 2 ml. of 2 mM-di-isopropyl phosphorofluoridate in 25 mM-tris–HCl buffer, pH 7·6, and 2 ml. of 25 mM-tris–HCl buffer, pH 7·6, were added. Then 4 ml. of this solution was added to 2 ml. of F-actin (2 mg./ml.) in 7·5 mM-$MgCl_2$–25 mM-tris–HCl buffer, pH 7·6. Then 3 ml. of the solution of the complex was pipetted into the viscometer, a small drop of octan-2-ol added and the viscosity measured before and after the addition of 0·075 ml. of 0·1 M-ATP.

Succinylation. Subfragment 1 (10–15 mg./ml.) was treated with succinic anhydride, at concentrations equivalent to 20–25 times the molar concentration of lysine present, under the conditions described by Habeeb, Cassidy & Singer (1958).

Amino acid analysis. Solutions of subfragment 1 were dialysed exhaustively against water, 5–6 vol. of cold acetone was added and the precipitate was washed twice with acetone and twice with ether. Approx. 20 mg. of the air-dried protein was dissolved in 10 ml. of conc. HCl and 1 ml. of this solution together with 1 ml. of mM-norleucine solution were sealed in a heavy-walled Pyrex tube. Hydrolysis was carried out in duplicate according to the method of Moore & Stein (1963) for 24, 48, 72 and 96 hr. After removal of the HCl the hydrolysate was dissolved in 1·5 ml. of 50 mM-phosphate buffer, pH 6·8, and kept for 4 hr. at room temperature to oxidize any remaining cysteine to cystine. Analyses were carried out on the Beckman model 120B amino acid analyser by using the elution procedure of Moore, Spackman & Stein (1958) as modified for accelerated analysis by Spackman (1963). Norleucine, β-thien-2-yl-DL-alanine and α-amino-β-guanidinopropionic acid were used as internal standards (Bargetzi, Kumar, Cox & Neurath, 1963).

Materials. Salt-free twice-crystallized trypsin, salt-free thrice-crystallized chymotrypsin and the disodium salt of ATP were supplied by the Sigma Chemical Co., St Louis, Mo., U.S.A. ATP was converted into the tetrasodium salt and stored frozen as 50 mM solutions. Subtilisin was the product of the Teikoku Chemical Industry Co. Ltd., Osaka, Japan, sold under the trade name Nagarse. Thrice-crystallized soya-bean trypsin inhibitor was obtained from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

RESULTS

Fractionation and biological activity of the H-meromyosin digests

In preliminary attempts to produce fragments with different biological properties from H-meromyosin, the effects of digestion with trypsin, chymotrypsin and subtilisin on the Ca^{2+}-activated ATPase and actin combination were compared. As shown in Fig. 1, the results with each enzyme were

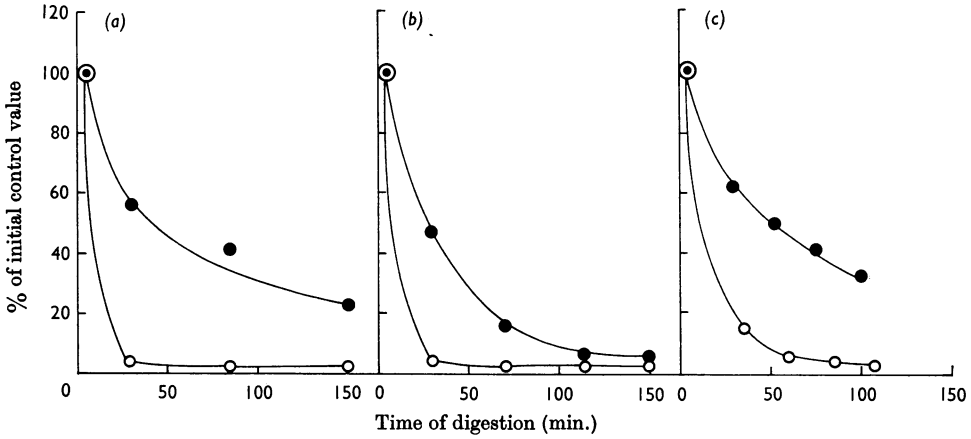

Fig. 1. Effect of proteolytic enzymes on the ATPase and actin-combining properties of H-meromyosin. H-meromyosin and enzymes were dissolved in 25mM-tris–HCl buffer, pH 7·6, in all cases, 4ml. portions of digest were removed at the times indicated and the reaction was stopped by the addition of 2ml. of 2mM-diisopropyl phosphorofluoridate for trypsin and chymotrypsin. (*a*) H-meromyosin (4·5mg./ml.) and trypsin (0·45mg./ml.); (*b*) H-meromyosin (4·7mg./ml.) and chymotrypsin (0·45mg./ml.); (*c*) H-meromyosin (4·7mg./ml.) and subtilisin (0·14mg./ml.). ●, ATPase activity; ○, actomyosin formation measured by viscosity fall with ATP.

rather similar. In general, actin combination as measured by the fall in viscosity produced when actin was added to the digest was more sensitive to digestion than was the enzymic activity. The preliminary experiments indicated that trypsin was at least as effective as the other enzymes in its apparent selective destruction of the viscosity effect. In view of this observation, and the specific nature of this enzyme, the course of tryptic digestion was studied in further detail by using lower concentrations of H-meromyosin and longer digestion times than were used in the original study (Mueller & Perry, 1962). After 90min. digestion of H-meromyosin (5mg./ml.) with trypsin (0·25mg./ml.), the ATPase activity was decreased by less than 50% of the original value, but the addition of ATP did not produce a measurable fall in viscosity of the complex formed when F-actin (2mg./ml. in 25mM-tris–hydrochloric acid buffer, pH 7·6) was added to the digest. Study of the progress of the digestion in the ultracentrifuge indicated that the original H-meromyosin boundary was replaced by at least two more slowly sedimenting boundaries (Fig. 2), as was reported by Mueller & Perry (1962).

Under the conditions of digestion described, about 60–65% of the original H-meromyosin was precipitable with 6% (w/v) trichloroacetic acid. The ATPase activity of the digest fell off rather quickly on standing, but was much more stable if the protein concentration was increased by precipitation with 55%-saturated ammonium sulphate. The procedure precipitated approx. 50% of the total nitrogen and no significant amounts of enzymic activity could be detected in the supernatant obtained. Control experiments on the solubility of trypsin–trypsin inhibitor complex alone in ammonium sulphate solutions at the concentration used for fractionating the digest suggested it would not be precipitated under these conditions. Nevertheless, from electrophoretic and DEAE-cellulose chromatography studies of the fraction precipitated by 55%-saturated ammonium sulphate there was some evidence that not all of the complex was removed when digests were treated in this way.

Chromatography of the 55%-saturated ammonium sulphate precipitate on Sephadex G-200 usually fractionated the digest into four main peaks, although resolution depended on the concentration of protein applied to the column (cf. Figs. 3 and 4). The second peak, representing slightly retarded material, was the most striking feature of the elution pattern and was the only fraction with ATPase activity (Fig. 3).

Although complex-formation on the addition of actin to the H-meromyosin digest could not be detected by viscosity changes on the addition of ATP, ultracentrifuge studies similar to those reported by Mueller & Perry (1962) indicated that this occurred. Attention was directed particularly to the fraction precipitated by 55%-saturated ammonium sulphate in which all the ATPase activity of the digest appeared to be localized. After the addition of F-actin to this fraction and subsequent centrifugation, the fastest component

in the digest (subfragment 1) rapidly sedimented and at least two major peaks of lower sedimentation coefficients could be recognized.

Chromatography on Sephadex G-200 of the 55%-saturated ammonium sulphate fraction before and after the addition of F-actin and centrifugation for 3·25hr. at 100000*g* indicated that the actin-combining fraction (subfragment 1) was localized in peak 2 (Fig. 4). After centrifugation with F-actin no ATPase activity was detected in the supernatant, strongly suggesting that all the ATPase activity was associated with the actin-combining fraction.

In addition to dissociation by ATP, the actin–subfragment 1 complex could be dissociated by pyrophosphate in the presence of Mg^{2+}. As pyrophosphate is not hydrolysed in the system and the ATPase activity of the subfragment isolated was usually considerably higher than that obtained with the ATP method (Mueller & Perry, 1962), a procedure employing pyrophosphate was developed for the routine preparation of subfragment 1 by actin combination (see the Methods section).

About 95% or more of subfragment 1 prepared by actin combination moved on Sephadex G-200 as a single peak that was eluted in the same position as peak 2 obtained on chromatography of the whole digest (Fig. 5). The small amount of material

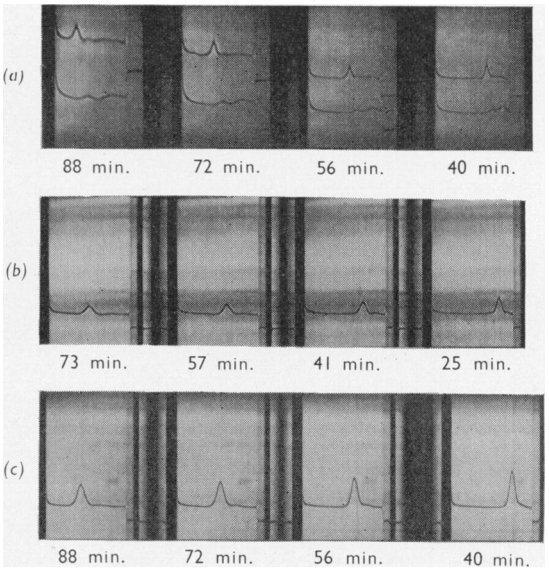

Fig. 2. Ultracentrifugation of H-meromyosin digest and subfragment 1. (*a*) H-meromyosin (5mg./ml. in 25mM-tris–HCl buffer, pH7·6) digested with 0·1vol. of trypsin (2·5mg./ml. in mN-HCl). After 78min. 0·2vol. of trypsin inhibitor (2·5mg./ml. in 25mM-tris–HCl buffer, pH7·6) was added. Ultracentrifugation conditions were: 56000rev./min.; bar angle, 55° (40 and 56min.) or 45° (72 and 88min.); temperature, 18·3°; double-sector cell with the upper boundary representing control H-meromyosin solution containing trypsin with inhibitor added at zero time. (*b*) Peak 2 separated by gel filtration on Sephadex G-200 of 55%-saturated $(NH_4)_2SO_4$ precipitate of the tryptic digest of H-meromyosin. Ultracentrifugation conditions were: 59780rev./min.; bar angle, 70°; temperature, 20°; 8·0mg./ml. in 0·15M-KCl–20mM-tris–HCl buffer, pH7·6. (*c*) Subfragment 1 separated by actin combination. Ultracentrifugation conditions were: 59780rev./min.; bar angle, 70°; temperature, 20°; 11·2mg./ml. in 0·15M-KCl–20mM-tris–HCl buffer, pH7·6.

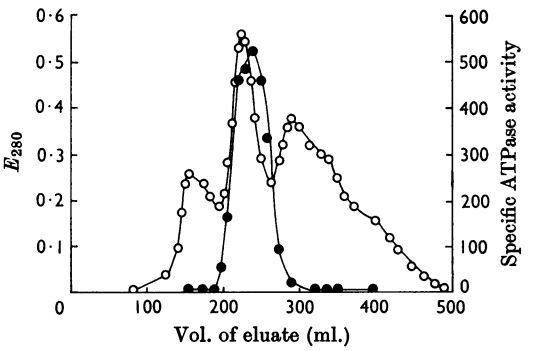

Fig. 3. Gel filtration on Sephadex G-200 of fraction precipitated from 90min. tryptic digest of H-meromyosin by 55%-saturated $(NH_4)_2SO_4$. A 4ml. sample of protein solution (E_{280} 20) was applied to a column (42cm. × 3·8cm.) equilibrated against 25mM-tris–HCl buffer, pH7·6, and protein was eluted with the same buffer at a flow rate of 8ml./hr. ○, E_{280}; ●, ATPase activity.

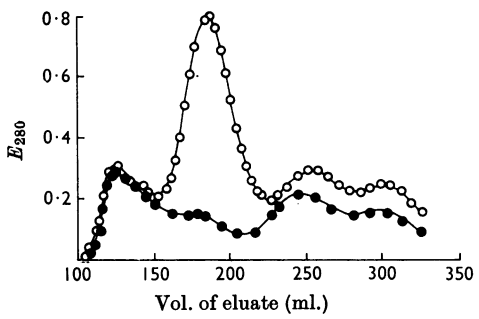

Fig. 4. Gel filtration on Sephadex G-200 of the 0–55%-saturated $(NH_4)_2SO_4$ fraction of the tryptic digest of H-meromyosin before and after the addition of F-actin. Conditions for gel filtration were as given for Fig. 2 on a column (34cm. × 4cm.) of Sephadex G-200 and elution was carried out with 25mM-tris–HCl buffer, pH7·6. ○, Whole 55%-saturated $(NH_4)_2SO_4$ precipitate (E_{280}16) (5ml.); ●, 55%-saturated $(NH_4)_2SO_4$ precipitate (E_{280}16) (5ml.) after the addition of 8ml. of F-actin solution (6mg./ml.) and centrifugation for 3·25hr. at 100000*g*, the supernatant being applied.

unretarded on Sephadex G-200, usually less than 5–6% of the total protein applied, was considered to represent aggregated subfragment. In the ultracentrifuge the subfragment-1 preparations obtained by actin combination were identical with those obtained by separating peak 2 from digests by gel-filtration procedures.

ATPase activity of subfragment 1

Activation by Ca^{2+}. When precautions were taken to avoid storage of preparations of subfragment 1 in dilute solution, the specific activity of the ATPase in the presence of Ca^{2+} (see the Methods section) was always greater than that of the H-meromyosin from which it was prepared. The precise percentage increase was somewhat variable, as shown in Table 1, in which the results have been obtained by assays carried out on pooled whole fractions of subfragment 1 isolated by the actin-combination and gel-filtration techniques. It was consistently noted that certain fractions of the subfragment-1 peak obtained on Sephadex G-200 possessed enzymic activities considerably higher than those of the pooled fractions from which they were obtained. In some preparations, fractions possessing a specific ATPase activity of 500–530 were obtained from H-meromyosin of original specific activity 220–270.

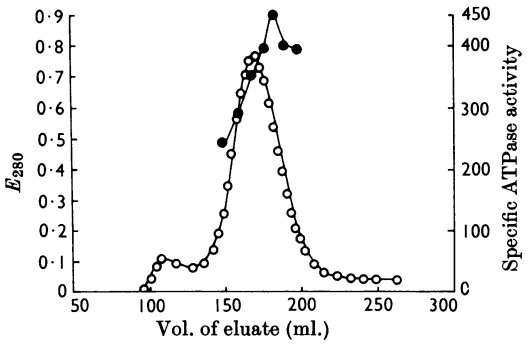

Fig. 5. Gel filtration on Sephadex G-200 of subfragment 1 isolated by actin combination. Subfragment 1 was isolated from a 90 min. tryptic digest as described in the Methods section. A 7 ml. sample of protein solution (E_{280} 5·88) was applied to a column (33 cm. × 3·7 cm.) equilibrated against 25 mM-tris–HCl buffer, pH 7·6. Protein was eluted with the same buffer. ○, E_{280}; ●, ATPase activity.

Subfragment 1 isolated by the Sephadex method was invariably activated by phenylmercuric acetate when assays were carried out at ionic strength greater than *I* 0·3. The extent of activation, as is often found with myosin, depended on the preparation and on its age. As has been reported for H-meromyosin by Perry & Cotterill (1965*a*), the activation by phenylmercuric acetate only occurred at ionic strength greater than 0·2 M-potassium chloride. In the absence of added potassium chloride, all concentrations of phenylmercuric acetate used caused marked inhibition (Fig. 6). In this behaviour subfragment 1 resembled H-meromyosin rather than myosin, the ATPase of which is activated by phenylmercuric acetate at somewhat lower ionic strengths (Perry & Cotterill, 1965*a*). Maximal activation in 0·3 M-potassium chloride occurred at concentrations of 1·7–2·4 moles of phenylmercuric acetate/10^5 g. of protein with subfragment 1, trypsin-prepared H-meromyosin and myosin.

The effects of phenylmercuric acetate on subfragment 1 isolated by the actin-combination method were much more variable. In only a few cases was activation obtained under conditions that

Table 1. *Comparison of ATPase activities of subfragment* 1 *and the H-meromyosin preparation from which it was derived*

The values given for subfragment 1 isolated by gel filtration represent the specific activity of the pooled fractions of the active peak. The assays were carried out in 5 mM-$CaCl_2$–5 mM-ATP–57·5 mM-tris–HCl buffer, pH 7·6.

H-meromyosin prep. no.	Method of isolation of subfragment 1	ATPase activity (μg. of P/mg. of N/min.) H-meromyosin	Subfragment 1	Increase (%)
19	Actin combination	220*	338	54
24	Actin combination	168*	300	78
27	Gel filtration	297‡	357	20
28	Actin combination	310*	380	23
32	Actin combination	163*	200	23
34	Gel filtration	282	415	47

* H-meromyosin was put through the entire isolation procedure.

‡ H-meromyosin was not submitted to gel filtration, but was dialysed in the same vessel as subfragment 1 before enzymic assay.

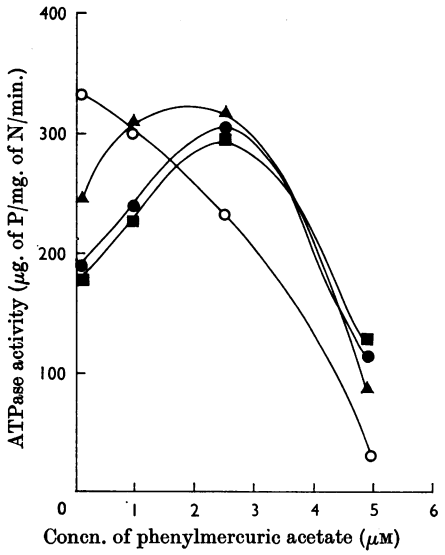

Fig. 6. Effect of KCl on the activation of the ATPase of subfragment 1 by phenylmercuric acetate. All assays were carried out in 2ml. of 5mM-ATP–5mM-$CaCl_2$–57·5mM-tris–HCl buffer, pH 7·6, containing 0·206mg. of protein and the KCl and phenylmercuric acetate concentrations indicated. ○, No added KCl; ▲, 0·3M-KCl; ●, 0·4M-KCl; ■, 0·5M-KCl.

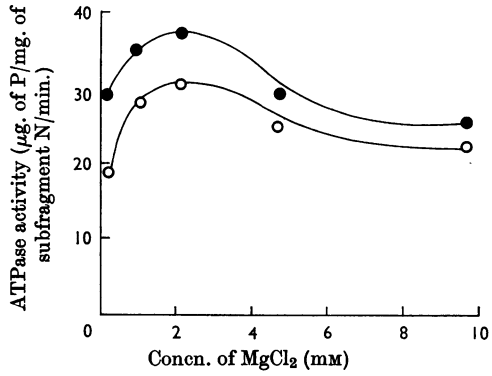

Fig. 7. Effect of Mg^{2+} on the ATPase activity of the actin–subfragment 1 complex. Assays were carried out in 2ml. of 2·5mM-ATP–5mM-mercaptoethanol–25mM-tris–HCl buffer, pH 7·6, and 0·4ml. of enzyme solution in 25mM-tris–HCl buffer, pH 7·6, made up as indicated. ○, Subfragment 1 (2·75mg./ml.) and F-actin (1·38mg./ml.); ●, subfragment 1 (2·75mg./ml.) and F-actin (2·75mg./ml.).

gave the effect with preparations isolated by the Sephadex method. In these cases maximal activation occurred with 0·8–0·96mole of phenylmercuric acetate/10^5g. of protein.

The Ca^{2+}-activated ATPase of subfragment 1 was usually, although not with all preparations, activated by *N*-ethylmaleimide. The extent of activation tended to be lower than that reported for H-meromyosin and myosin, and only occurred at potassium chloride concentrations above 0·3M.

Activation by Mg^{2+}. Previous studies (Leadbeater & Perry, 1963) indicated that the Mg^{2+}-activated ATPase of myosin in the presence of actin was sensitive to the tryptic digestion of myosin to H-meromyosin. The results obtained with H-meromyosin in these studies were somewhat variable, but although the activation by Mg^{2+} was often much diminished it usually survived to some extent.

Subfragment 1, purified by Sephadex chromatography or actin combination, had very low enzyme activity in 2·5mM-ATP–25mM-tris–hydrochloric acid buffer, pH 7·6, in the absence of bivalent activators. This amounted to about 5% of the specific activity observed when calcium chloride (final concn. 2·5mM) was added to the system. The addition of actin in the absence of added bivalent ions produced a slight increase in activity that rose as the actin/subfragment 1 ratio increased (Fig. 7), but in general the effect of actin was somewhat less than is obtained with myosin under similar conditions. In contrast with the effects obtained with Ca^{2+}, over the Mg^{2+} concentration range 0–10mM, the maximal activation of the ATPase of the actin–subfragment 1 complex, obtained with 1–2mM-Mg^{2+}, was usually only 25–30% greater than that obtained in the absence of bivalent cations (Fig. 8). The marked contrast in the behaviour of myosin under similar conditions is shown in Fig. 8, which also illustrates the difference in the effects of Ca^{2+} and Mg^{2+} on the ATPase of the actin–subfragment 1 complex at low ionic strength.

In view of the report (Gaetjens, Therattil-Antony & Barany, 1964) that, under certain conditions of disulphide exchange, myosin ATPase no longer shows activation by Mg^{2+} in the presence of actin, it was considered possible that some exchange or oxidation of thiol groups associated with the active centre of subfragment 1 might be responsible for this effect. To eliminate this possibility subfragment 1 was prepared in the presence of 10mM-mercaptoethanol to maintain all thiol groups fully reduced. This procedure increased the APTase activity but did not change the pattern of response to Mg^{2+}, indicating that this was not the explanation for the loss in activation by Mg^{2+} (Fig. 7).

Enzymic heterogeneity. It was noted in studies on the distribution of ATPase activity after Sephadex G-200 chromatography of digests that the peak of specific APTase activity was sometimes slightly retarded compared with the main protein peak eluted and with which the actin-combining activity

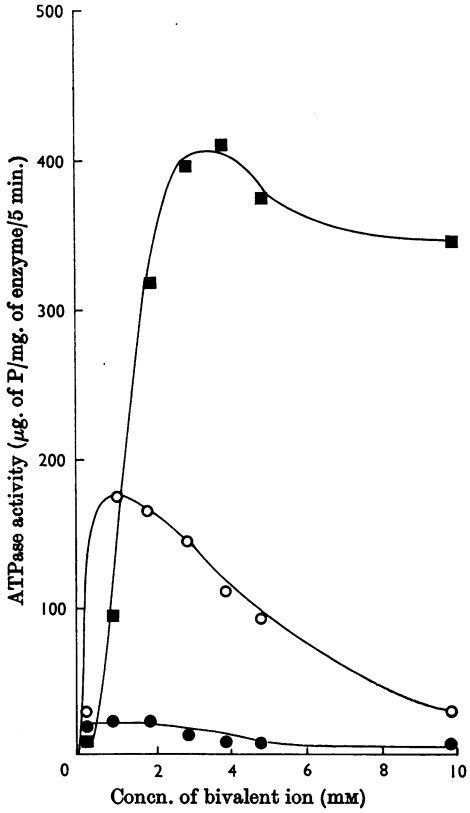

Fig. 8. Comparison of the effects of Ca^{2+} and Mg^{2+} on the ATPase activity of the actin–subfragment 1 complex. Incubations were carried out in the absence of mercaptoethanol but otherwise as described for Fig. 7, with the final protein concentrations indicated. ●, Subfragment 1 (0·3 mg./ml.) and actin (0·15 mg./ml.), Mg^{2+} present; ○, myosin (0·3 mg./ml.) and actin (0·15 mg./ml.), Mg^{2+} present; ■, subfragment 1 (0·075 mg./ml.) and actin (0·0375 mg./ml.), Ca^{2+} present.

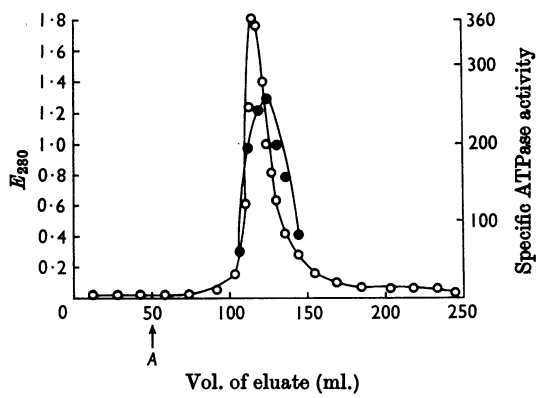

Fig. 9. Ion-exchange chromatography of subfragment 1 isolated by actin combination. A 20 ml. sample of protein solution (E_{280} 3·18) in 25 mM-tris–HCl buffer, pH 8·2, was applied to a column (12 cm. × 2·2 cm.) of DEAE-cellulose (Whatman grade C1). A linear gradient to 0·3 M-KCl–25 mM-tris–HCl buffer, pH 8·2, was applied at *A*, mixing chambers of 150 ml. capacity being used. ATPase activity was assayed at pH 8·2, but otherwise as described in the Methods section. ○, E_{280}; ●, ATPase activity.

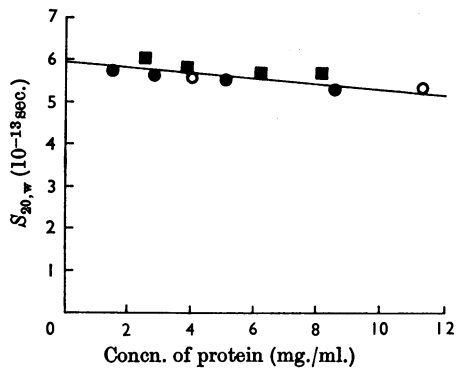

Fig. 10. Effect of concentration on the sedimentation coefficient of subfragment 1. The conditions were: 59 780 rev./min.; temperature, 20°; 0·15 M-KCl–20 mM-tris–HCl buffer, pH 7·6. ○ and ●, Preparations isolated by actin combination; ■, preparation isolated by gel filtration.

was associated (see Figs. 3 and 5). When subfragment 1 was isolated by actin combination and then chromatographed on DEAE-cellulose, it was eluted at 0·10 M-potassium chloride–25 mM-tris–hydrochloric acid buffer, pH 8·2, as one fairly symmetrical peak (Fig. 9). Nevertheless, fractions with the highest specific ATPase activity did not coincide with the fractions of highest protein concentration. Invariably the peak of specific ATPase activity was found in the trailing edge of the eluted material.

Sedimentation and diffusion studies

Preparations of subfragment 1, whether made by the Sephadex or actin-combining procedures, gave a consistent picture (Fig. 2) in the ultracentrifuge of one main component with a small amount of a slower component (less than 10%). Despite the enzymic evidence, careful study of the sedimentation and diffusion characteristics gave no indication of heterogeneity of the main component. The sedimentation coefficient was not very concentration-dependent and on extrapolation gave $S^0_{20,w}$ $5{\cdot}95 \times 10^{-13}$ sec. (Fig. 10), which is identical with that reported by Mueller & Perry (1962) for subfragment 1. The diffusion coefficient appeared to be somewhat more dependent on concentration

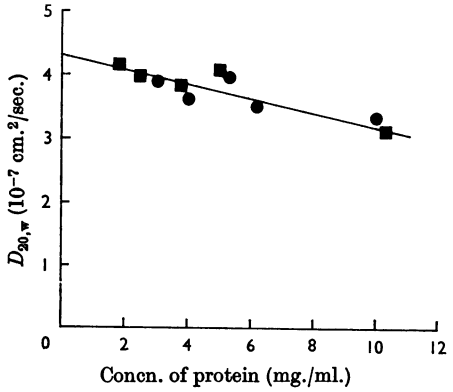

Fig. 11. Effect of concentration on the diffusion coefficient of subfragment 1 isolated by actin combination. The conditions were: 2531 rev./min.; temperature, 20°; 0·15M-KCl–20mM-tris–HCl buffer, pH 7·6. ●, Preparation isolated by actin combination; ■, preparation isolated by gel filtration.

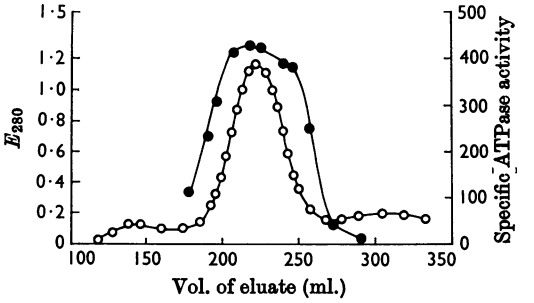

Fig. 12. Gel filtration on Sephadex G-200 of fraction precipitated after chymotryptic digestion by 55%-saturated $(NH_4)_2SO_4$. A 70ml. sample of H-meromyosin (5·0mg./ml.) was digested with 0·1vol. of chymotrypsin (2·5mg./ml. in mN-HCl) for 30min. at 25°. The reaction was stopped by adding mM-di-isopropyl phosphorofluoridate. The fraction of the digest precipitated by 55%-saturated $(NH_4)_2SO_4$ was equilibrated with 25mM-tris–HCl buffer, pH 7·6, and applied to a column (42cm. × 3·7cm.) of Sephadex G-200. ○, E_{280}; ●, ATPase activity.

(Fig. 11). Extrapolation of the line of best fit to zero concentration gave $D^0_{20,w}$ $4{\cdot}33 \times 10^{-7}$cm.^{2}/sec. Assuming a partial specific volume of 0·742 (Mueller, 1965), these values correspond to a molecular weight of 129000.

The small amounts of the minor component that were present in all preparations studied, whether prepared by gel-filtration or actin-combination methods, did not permit accurate determination of its sedimentation coefficient. In general, the sedimentation velocity of this component was similar to that reported by Mueller & Perry (1962) for subfragment 2. The significance of this consistent contaminant of subfragment preparations is not clear, but the fact that it was present in the preparations obtained by actin combination suggests that it did in fact combine with this protein.

On ultracentrifugation in 25mM-tris–hydrochloric acid buffer, pH 7·6, after succinylation and removal of excess of reagent, subfragment 1 sedimented as a disperse heterogeneous system. In view of the fact that myosin sediments as a single peak under these conditions this result indicated that the subfragment had probably dissociated into a number of fragments of different size.

Subfragment 1 by chymotryptic digestion

Limited studies on the further digestion of trypsin-prepared H-meromyosin with chymotrypsin indicated that a subfragment-1-like component was produced. Fig. 12 illustrates the results of Sephadex chromatography on such a digest otherwise treated as described in Fig. 3. All ATPase activity was associated with the main component, which was slightly retarded on Sephadex G-200 as was the case with tryptic digests. In chymotryptic digests an unretarded fraction was obtained on Sephadex G-200 as was obtained with tryptic digests of H-meromyosin. In the chymotryptic digest the unretarded material appeared to be derived from the H-meromyosin digest, whereas in the tryptic digest it was not possible to assess the contribution, if any, of the trypsin–trypsin inhibitor complex to the unretarded fraction. The active fragment isolated by actin combination from the 90min. digest had a sedimentation coefficient of $5{\cdot}75 \times 10^{-13}$sec.

Similar preparations of subfragment 1 were obtained if chymotrypsin-prepared H-meromyosin was subsequently digested with chymotrypsin.

Amino acid analysis

Although the content of residues such as tyrosine, serine, histidine and threonine was remarkably constant in subfragment-1 preparations, the lysine, phenylalanine, aspartic acid and ammonia values were somewhat more variable, suggesting that slight differences in content of these latter residues might exist between different preparations (Table 2). It was unlikely that these variations were due to analytical errors because for a given subfragment-1 preparation the results were consistent when duplicate analyses were carried out on 24, 48, 72 and 96hr. hydrolysates.

Averaging the analytical data for each residue indicated significant differences between the composition of subfragment 1 and H-meromyosin. The lysine and glutamic acid contents of the former were

Table 2. *Amino acid composition of subfragment* 1

Subfragment 1 preparations obtained by the actin-combination method. The yields of serine, threonine, tyrosine, aspartic acid and glutamic acid were extrapolated to zero time to correct for hydrolytic destruction. An average of the 24, 48, 72 and 96 hr. hydrolysate values was used for the remaining residues.

	Composition of subfragment 1 (moles/10^5 g.)				Composition of H-meromyosin* (moles/10^5 g.)
Residue	Prep. 1	Prep. 2	Prep. 3	Average	
Lys	67	74	71	71	86
His	14	15	15	15	14
NH_3	88	93	97	93	90
Arg	31	34	32	32	34
Asp	87	91	95	91	82
Thr	49	50	51	50	44
Ser	43	42	43	43	39
Glu	122	126	129	126	137
Pro	29	29	32	30	32
Gly	54	55	57	55	50
Ala	65	69	67	67	73
CyS	10	9	11	10	7·4
Val	49	49	54	51	48
Met	27	29	30	29	26
Ile	50	51	55	52	44
Leu	70	73	75	73	73
Tyr	31	31	32	31	21
Phe	44	45	50	46	36

* Lowey & Cohen (1962).

lower, whereas the aspartic acid, cysteine and particularly the phenylalanine and tyrosine contents were higher. The content of tyrosine was greater by almost 50% and represented the greatest difference between the amino acid composition of the subfragment 1 and H-meromyosin.

DISCUSSION

Subfragment-1 preparations isolated either by gel filtration or by actin combination from prolonged digests of H-meromyosin possessed an $S^0_{20,w}$ value identical with that reported by Mueller & Perry (1962) for the main subfragment isolated after various intervals of digestion. The molecular weight of 129 000 falls in the range obtained by sedimentation-equilibrium studies on subfragment 1 obtained by other workers, although the longest digestion times by these investigators were appreciably shorter than those used in our standard method of preparation.

The lack of coincidence of the protein peak obtained on chromatography of subfragment 1 and the peak of specific ATPase activity suggests either that there exists a population of fragments of different ATPase activities, or that if there is one fragment of fixed enzymic activity it is somewhat smaller in size and more acidic in nature than the bulk of the protein that combines with actin. Although it seems unlikely on quantitative grounds alone that the minor slow component present in subfragment preparations is responsible for all the ATPase activity, the possibility that it is enzymically active has not been completely excluded. It is noteworthy that in Mueller's (1965) study this minor component appears consistently in his subfragment-1 preparations obtained after short digest times.

A more likely explanation of the apparent heterogeneity is that subfragment-1 preparations may consist of a population of fragments of similar molecular sizes with perhaps some variation in biological activity so far as relative ability to combine with actin and to hydrolyse ATP are concerned. This could be due to the digestive process itself, or could arise as a consequence of changes in the relative stability of these properties during the preparative procedures.

Final explanation of the findings must await further work, but it may be concluded that, under the conditions of digestion used, clear evidence has not yet been obtained for the independent association of either biological property with any protein fragment. The findings of Nankina *et al.* (1964) are of considerable interest, but the evidence of non-formation of an actin complex by the enzymically active fragment isolated by these workers was based on the inability to show a change in birefringence

when ATP was added to a solution containing subfragment 1 and F-actin. The molecular parameters responsible for birefringence are in some circumstances similar to those governing viscosity, and in view of the small changes in the latter, when subfragment 1 interacts with F-actin, the findings of Nankina *et al.* (1964) need to be confirmed by a more sensitive method of observing interaction.

The characteristics of the enzymic activity of subfragment 1 are similar to those of trypsin-prepared H-meromyosin, and as is the case with the latter enzyme a higher ionic strength is required to demonstrate activation by thiol reagents. The most striking difference from both H-meromyosin and myosin is the inability of the subfragment ATPase to be appreciably activated by Mg^{2+} in the presence of F-actin, despite the fact that under similar conditions the specific activity of the Ca^{2+}-activated enzyme is several times greater than that of myosin. The precise explanation of this effect is not clear, but the results suggest that during the preparation of subfragment 1, and to some extent during H-meromyosin preparation by tryptic digestion (Leadbeater & Perry, 1963), proteolytic attack has occurred in the region of the sites on the myosin molecule required for the interaction with actin that results in activation by Mg^{2+}. Whatever the explanation, the observation is good evidence that the site on the actin molecule involved in forming the actomyosin complex is not identical with that concerned in promoting activation by Mg^{2+} of myosin ATPase (Perry & Cotterill, 1965*b*).

The increases in specific activity of subfragment 1, which in some fractions were greater than 100% when related to E_{280}, would be even greater if based on total nitrogen, for the content of aromatic amino acids was significantly higher than in H-meromyosin. This fact indicates that subfragment-1 formation had involved extensive removal of enzymically inert material from the H-meromyosin molecule and fits in well with the molecular-weight determinations. Any deductions about the extent of digestion made on the basis of recovery of enzyme activity will tend to be minimal, for the instability of the ATPase in solutions of low protein concentration will tend to indicate that less inert material has been digested away than has in fact occurred.

In general, with exception of lysine, tyrosine and phenylalanine, the amino acid composition of subfragment 1 was generally similar to that of H-meromyosin itself. This finding supports the view that repeating sequences occur along the polypeptide chains of which the myosin molecule is composed (Perry & Landon, 1964; Perry, 1965). From the 50% increase in tyrosine content it follows that, if all of this amino acid were concentrated in the part of the H-meromyosin molecule that remains intact during the digestion and gives rise to the subfragment 1, one-third of the molecule would need to be removed to give the analytical values obtained. Comparison of the tyrosine contents of L-meromyosin and H-meromyosin indicates, however, that this amino acid is distributed throughout the molecule, although some concentration does occur in the H-meromyosin portion. In these circumstances it would be expected that much more than one-third of the H-meromyosin molecule would have to be removed to produce a fragment of the tyrosine content associated with subfragment 1.

In view of these facts, we do not consider that our studies on subfragment 1 support the hypothesis of Young *et al.* (1965). The properties of the subfragment 1 obtained by our procedures are more readily explained if it is considered to be produced by extensive digestion of the H-meromyosin molecule leaving the thickened or globular portion of the original myosin molecule intact. Mueller (1965) concludes that 55–60% of the H-meromyosin gives rise to subfragment 1, for which he obtains molecular weights in the range 112000–170000, but does not completely exclude the possibility of two subfragment-1 molecules being produced/H-meromyosin molecule. The final answer to the problem of the relation of subfragment 1 to the H-meromyosin and myosin molecules clearly depends on the number of centres on the thickened part of the myosin molecule able to hydrolyse ATP and combine with actin. Evidence from the binding of pyrophosphate (Gergely, Martonosi & Gouvea, 1959) and of ATP (Nanninga & Mommaerts, 1960) suggest one for the former function. This situation would require that one subfragment-1 molecule would be obtained/H-meromyosin molecule and fits in well with the present results.

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