Studies on the Heterogeneity of Subfragment-1 Preparations

ISOLATION OF A NEW PROTEOLYTIC FRAGMENT OF THE HEAVY CHAIN OF MYOSIN

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1. The physical, chemical and enzymic properties of subfragment 1 prepared from myosin of rabbit skeletal muscle by using two different concentrations of insoluble papain were compared. 2. Subfragment 1 prepared by using a myosin/papain ratio of 2000:1 (by wt.) migrated on electrophoresis in non-dissociating conditions as a single enzymically active band. When prepared with a myosin/papain ratio of 200:1 the preparation consisted of two enzymically active components of slightly different electrophoretic mobility. 3. The two types of preparation were obtained in similar yield and possessed similar specific adenosine triphosphatase activities when determined in the presence of Ca$^{2+}$. 4. Gel electrophoresis in the presence of 8M-urea showed that both preparations contained three light components. The component of molecular weight 15500 was apparently identical with one of the light-chain components of myosin (M$_1$). The other two light-chain components of subfragment 1 were not identical with any of the light-chain components of myosin. 5. The heavy-chain fraction of subfragment 1 prepared by using low concentrations of papain dissociated into components with molecular weights of 87000, 69000 and 26000 on electrophoresis in sodium dodecyl sulphate. The heavy-chain fraction of subfragment 1 prepared by using higher concentrations of papain contained components with molecular weights of 69000 and 53000 and relatively increased amounts of the component of molecular weight 26000. 6. The isolated 26000 dalton component had an amino acid composition similar to that of the heavy-chain fraction of subfragment 1 and contained 3-methylhistidine and mono- and tri-$N^\alpha$-methyl-lysine. It was homogeneous on electrophoresis in the presence of sodium dodecyl sulphate but gave two bands on electrophoresis in 8M-urea.

Earlier investigations have shown that heavy meromyosin can be further digested with trypsin to produce a protein fragment possessing ATPase† and actin-combining activities, which is termed subfragment 1, of molecular weight about one-quarter of that of the original myosin molecule (Mueller & Perry, 1961, 1962). Studies of subfragment 1 produced by trypsin digestion led to uncertainties about how many molecules were derived from each myosin molecule, probably owing to the fairly extensive proteolysis that occurred under the conditions of its preparation (Mueller, 1965; Young et al., 1965; Jones & Perry, 1966). The observation that digestion with insoluble papain produced subfragment 1 from myosin in a single step (Kominz et al., 1965) was subsequently exploited by Lowey et al. (1969) to examine the formation of subfragment 1 in more detail. They confirmed that two molecules of subfragment 1 were formed per molecule of myosin, as would be expected from the double-headed appearance of the myosin molecule in the electron microscope (Slayter & Lowey, 1967).

Although it is widely accepted that a subfragment-1 molecule is derived from each of the globular heads of myosin, it is a matter for debate whether the two molecules produced are identical. Myosin from rabbit skeletal muscle contains a total of four molecules of light-chain fraction per molecule and electrophoretic and analytical evidence indicate that the fraction is composed of three different molecular species (Weeds & Lowey, 1971). Clearly there are difficulties in producing two identical subfragment-1 molecules if all three types of light chain are integral parts of the myosin molecule and are distributed between the two myosin heads. The problem is further complicated if skeletal-muscle myosin is composed of a mixture of isoenzymes (Trayer & Perry, 1966). For example, myosin isoenzymes might contain different light components although each type of myosin molecule possessed heads of identical structure. In this case different subfragment-1 molecules would arise from different isoenzyme forms and not from a single myosin molecule.
A number of workers have reported subfragment-1 preparations obtained by trypsin or papain digestion to be homogeneous on the basis of sedimentation-velocity studies, but this method does not have the resolution necessary to detect heterogeneity of the type discussed above. Heterogeneity of subfragment 1 prepared by trypsin digestion has been indicated by electrophoretic studies (Nauss et al., 1969) and of subfragment 1 prepared by papain digestion by chromatography on DEAE-cellulose (Lowey et al., 1969). Such observations, however, need not necessarily indicate structural heterogeneity in the original myosin molecule, but could arise from the difficulties in producing a unique proteolytic fragment from a larger homogeneous molecule containing a number of susceptible bonds.

To evaluate this problem and to attempt to decide whether subfragment-1 molecules could be produced that reflected structural differences between myosin molecules or between the globular heads of a given myosin molecule, a study has been made of the effect of papain concentrations on subfragment-1 formation. This investigation has led to the characterization of a proteolytic fragment derived from the heavy chain of myosin that has not been previously described. Some aspects of the work have been briefly reported elsewhere (Perrie et al., 1969; Stone & Perry, 1972).

Materials and Methods

Materials

Crystalline papain was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A., and N-benzoyl-L-arginine ethyl ester hydrochloride by Mann Research Laboratories, Orangeburg, N.Y., U.S.A.

Preparation of myosin

Myosin was prepared from rabbit skeletal muscle by the method of Trayer & Perry (1966). All solutions used in the preparation contained 10 mM-2-mercaptoethanol (Perrie & Perry, 1970).

Preparation and assay of insoluble papain

Water-insoluble papain was prepared by coupling crystalline papain to diazotized p-aminobenzylcellulose as described by Lowey et al. (1969).

The enzymic activity of soluble and insoluble papain was measured by the rate of hydrolysis of N-benzoyl-L-arginine ethyl ester hydrochloride at pH 6.0 and 25°C by the pH-stat method (Lowey et al., 1969). The reaction mixture, which contained 5 ml of 25 mM-N-benzoyl-L-arginine ethyl ester hydrochloride, 5 mM-cysteine, 2 mM-EDTA and the appropriate amount of papain or cellulose–papain suspension, was titrated with 0.1 M-KOH.

The concentration of papain in the insoluble suspension was calculated as an equivalent concentration of soluble papain from the specific activity of the insoluble papain, assuming that the specific activity of soluble papain was unchanged by coupling to cellulose. For this calculation the specific activity of soluble papain was taken as 15 μmol of substrate hydrolysed/min per mg of enzyme protein. Some variation occurred between batches of soluble papain, but this was the average value. The amount of soluble papain calculated in this way as being equivalent to the insoluble papain was used to define the papain/myosin ratio used for digestion.

Subfragment 1

A freshly prepared solution of myosin (15–20 mg/ml) in 0.5 M-KCl, 5 mM-cysteine, 2 mM-EDTA, 15 mM-sodium phosphate buffer, pH 7.0, was stirred for 10 min at 25°C with a known amount of insoluble papain sufficient to give a myosin/equivalent papain ratio of either 200:1 or 2000:1. The insoluble papain was removed by centrifugation at 3000 g for 5 min at 4°C and the supernatant was further clarified by centrifugation at 70000 g for 30 min. The supernatant was then dialysed overnight against 10 vol. of 10 mM-2-mercaptoethanol, 5 mM-sodium phosphate buffer, pH 7.0, and the material precipitated after dialysis was removed by centrifugation at 70000 g for 45 min.

The supernatant was concentrated by ultrafiltration and fractionated on a column (2.5 cm × 100 cm) of Sephadex G-200 equilibrated with 10 mM-2-mercaptoethanol, 25 mM-Tris–HCl, pH 7.6, at 4°C. The second of the three peaks eluted under these conditions represented subfragment 1, and fractions were pooled for subfragment-1 preparations (Young et al., 1965; Lowey, 1968).

For the experiments in which the yields were determined the procedure outlined above was carried out with the modification that the 25 mM-Tris–HCl buffer, pH 7.6, used for gel filtration on Sephadex G-200 was replaced by 20 mM-sodium phosphate buffer, pH 7.0, to facilitate the determination of total nitrogen on the fractions. At each stage of the preparation, samples were removed for nitrogen determination and the volumes of the various fractions were recorded to correct for volume changes that occurred on dialysis and on redissolving the fraction precipitated by dialysis.

Determinations of ATPase

Specific ATPase activity was determined by incubation of 0.2 mg of enzyme in 2 ml containing 5 mM-CaCl₂, 5 mM-Tris–ATP, 25 mM-Tris–HCl buffer, pH 7.6, for 5 min at 25°C. The reaction was stopped by
the addition of 1 ml of 15% (w/v) trichloroacetic acid and the P1 liberated was determined by the procedure of Fiske & SubbaRow (1925).

**Protein determination**

Protein concentration was determined by an ultracentrifuge method of determination of N involving nesslerization (Strauch, 1965), assuming a N content of 16%.

**Polyacrylamide-gel electrophoresis**

(a) Vertical polyacrylamide-gel electrophoresis of subfragment 1 in non-dissociating conditions was performed essentially as described by Akroyd (1967). The gels contained 8\% (w/v) Cyanogum 41, 40\% (v/v) glycerol, 20\text{mM-Tris} - 125\text{mM-glycine buffer, pH8.6.} The running buffer was 20\text{mM-Tris} - 125\text{mM-glycine, pH8.6.}

(b) Vertical polyacrylamide-gel electrophoresis of urea-treated myosin and subfragment 1 was carried out as described by Perrie & Perry (1970). The gels contained 10\% (w/v) Cyanogum 41, either 8\%-urea or 40\% (w/v) glycerol, and 20\text{mM-Tris} - 125\text{mM-glycine buffer, pH8.6.} The running buffer was 20\text{mM-Tris} - 125\text{mM-glycine, pH8.6.}

(c) Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out on gels containing 10\% (w/v) acrylamide, 0.1\% sodium dodecyl sulphate and 0.1\text{M-sodium phosphate buffer, pH7.0,} as described by Weber & Osborn (1969).

Urea and glycerol gels were stained for protein with Amido Black (Davies, 1964) and for ATPase by the method of Wachstein & Meisel (1957). Sodium dodecyl sulphate gels were stained for protein with Coomassie Brilliant Blue R, Weber & Osborn (1969). Densitometric traces of stained gels were obtained by scanning with a Chromoscan J312 scanner (Joyce, Loebl & Co., Gateshead, U.K.).

**Amino acid analyses**

Amino acid analysis was carried out with the Beckman model 120B and Unichrom amino acid analysers. The amounts of amino acid were estimated by the height–width method of Spackman et al. (1958). Mono-N\textsuperscript{2}-methyl-lysine, tri-N\textsuperscript{2}-methyl-lysine and 3-methylhistidine were measured as described by Hardy et al. (1970).

**Determination of molecular weight by sedimentation-equilibrium studies**

These were performed in a Beckman model E analytical ultracentrifuge equipped with Rayleigh interference optics. Both the high-speed method of Yphantis (1964) and the intermediate-speed method of Chervenka (1970) were used. Interference patterns were measured with a micro-comparator. The natural logarithm of the fringe displacement was plotted against the square of the distance from the centre of rotation, and the slope of the lines was estimated by the method of least squares. A partial specific volume of 0.737, calculated from the amino acid composition of subfragment 1 as described by Cohn & Edsall (1943), was used for the determination of weight-average molecular weights by the method of Yphantis (1964).

**Results**

**Gel electrophoresis of subfragment 1 in non-dissociating conditions**

Unlike the myosin from which it was derived, heavy meromyosin subfragment 1 migrated to the cathode on electrophoresis on 8\% polyacrylamide gels containing 20\text{mM-Tris–glycine buffer, pH8.6,} in the absence of dissociating agents. Under these conditions subfragment 1 prepared at high papain concentration migrated as a broad band, which on close examination could be seen to represent two poorly resolved bands. Addition of 40\% (v/v) glycerol (Perrie & Perry, 1970) to the gel sharpened the bands and improved the resolution so that it became apparent that the electrophoretic pattern obtained with subfragment 1 was determined by the concentration of papain used during its preparation (Plate 1). Under the standard procedure described in the Materials and Methods section and at a papain/myosin ratio of 1:200, subfragment 1 invariably migrated in the glycerol–Tris–glycine system as two bands, which, although not well separated, were quite distinct. No other bands moving to the cathode could be detected and no significant amounts of material stayed at the origin (Plate 1). Both bands represented material with enzymic activity, for when the gels were stained for ATPase activity by the procedure of Wachstein & Meisel (1957) a double-band picture coincident with that obtained with the normal protein stain was obtained (Plate 1). Thus it appeared that the preparations made with high papain concentrations contained two components of slightly different electrophoretic mobility, both with ATPase activity.

When the weight ratio of papain used in the preparation was decreased the amount of the faster component fell until at the ratio of 1:2000 the subfragment migrated in the glycerol–Tris–glycine system, pH 8.6, as a single band corresponding to the slower of the two obtained with preparations made at higher concentrations of papain.

The presence of the two enzymically active components in subfragment 1 preparation was determined by the papain concentration rather than by the
time of exposure to the enzyme. As far as could be judged from the electrophoretograms, the faster component was present after 1 min digestion with papain in the ratio of 1 to 200 parts of myosin and there was no clear evidence that the relative proportions of fast and slow components changed with the progress of digestion. Even after prolonged digestion (100 min) with the low papain concentration only the slow-band component could be detected on electrophoresis.

Preliminary studies on subfragment 1 obtained by tryptic digestion of heavy meromyosin as described by Jones & Perry (1966) at a trypsin/myosin ratio of 1:20 indicated that this preparation also migrated as two bands on electrophoresis in the glycerol—Tris—glycine system.

Yields of subfragment 1 preparations

The two components of different electrophoretic mobility present in subfragment-1 preparations prepared by high papain concentrations could represent fragments derived from identical myosin molecules by different degrees of proteolysis. Alternatively, if myosin exists in isoenzyme forms the two components could be derived from myosin molecules of slightly different structure and which differ in their susceptibilities to digestion by papain. The yields of subfragment 1 obtained on preparation at high and low concentrations of papain were therefore estimated in an attempt to decide between these possibilities.

The results (Table 1) indicated that the yields of both preparations were similar. When myosin was digested with the high concentration of papain, 43% by weight of the myosin was converted into subfragment 1, whereas when low concentrations of papain were used the subfragment-1 fraction represented some 36% of the weight of the myosin molecule. Although the relative amounts of the two components obtained at high papain/myosin ratios were somewhat variable, in general there were always substantial proportions of both present. The slower component was usually present in slightly higher amounts than the faster.

The specific ATPase activity of the two types of subfragment-1 preparation were similar. Owing to the progressive inactivation of the ATPase of subfragment 1 during prolonged separation procedures, direct comparison of the purified preparations gave somewhat variable results but no consistent pattern was apparent. When the specific enzyme activity was determined indirectly by determining the total ATPase activity of the digest immediately after papain digestion and subsequently measuring the amount of subfragment 1 present by isolation, very similar values for both preparations were obtained (Table 2).

Gel electrophoresis of subfragment 1 in dissociating conditions

When subfragment 1 was subjected to electrophoresis on polyacrylamide gels in 20 mM-Tris—glycine buffer, pH 8.6, containing 8 mM-urea, three fairly fast bands were obtained. As is the case when myosin itself is subjected to electrophoresis under identical conditions, most of the applied protein remained at the origin. By analogy with myosin the protein remaining at the origin was presumed to represent the heavy-chain fraction of subfragment 1 and the bands migrating into the gel the light-chain components. Three bands were observed to move into the gel irrespective of whether high or low concentrations of papain were used. If the preparations were carefully handled and mercaptoethanol was present throughout few other bands were present. These bands were designated, in order of decreasing mobility, SM11, SM12 and SM13 (Plate 2, a and d). The three-band pattern was not changed if the subfragment-1 preparations were fully reduced with mercaptoethanol and carboxymethylated with iodoacetate before application to the polyacrylamide gel.

The fastest band present in subfragment 1, SM11, had the same electrophoretic mobility as M11, the

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Table 1. Distribution of nitrogen between fractions of papain digests of myosin

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Table 2. Ca²⁺-stimulated ATPase activities of subfragment-1 preparations

Activities are expressed as μg of P₁ liberated/min per mg of protein. The specific activities were determined on the whole digest as described in the Materials and Methods section. It was assumed that all ATPase activity was due to subfragment 1, the amount of which present was determined by isolation.

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fastest band present in myosin preparations when electrophoresis was carried out in 20 mM-Tris–glycine buffer, pH 8.6, containing 8 M-urea. The mobilities of the fastest band in subfragment 1 (SM1) and in myosin (M1) [the nomenclature of Perrie & Perry (1970) being used] were also identical when electrophoresis was carried out in 40% glycerol–20 mM-Tris–glycine buffer, pH 8.6, after prior treatment with urea (Perrie & Perry, 1970). The bands SM1 and MI, moved faster in 20 mM-Tris–glycine buffer, pH 8.6, containing 8 M-urea than the two bands MI and MI, respectively representing the light chain of intermediate electrophoretic mobility present in myosin (Perrie & Perry, 1970). On electrophoresis in 40% glycerol, however, the mobilities of bands SM1 and MI were relatively unaffected whereas those of bands MI and MI of myosin were much decreased (Plate 2).

The combined low-molecular-weight fraction of subfragment 1 had a similar solubility to the corresponding fraction isolated from myosin. It could be separated from the heavy-chain fraction by the procedure of Perrie & Perry (1970), although the yields were low with the relatively small amounts of subfragment 1 available. It was therefore more convenient to fractionate 50 mg batches of subfragment 1 into light- and heavy-chain fractions by gel filtration on Sephadex G-100 in the presence of 25 mM-Tris–HCl buffer, pH 7.6, containing 7 M-urea. The fraction represented by the retarded peak (Fig. 1), which contained the low-molecular-weight chains, was collected and any contaminating heavy-chain material removed by precipitation with 66% (v/v) ethanol.

The amino acid analysis of the total light-chain fraction of subfragment 1 isolated by gel filtration was similar to that of the total light-chain fraction of myosin and quite different from that of the heavy-chain fractions of myosin and subfragment 1 (Table 3).

Relationship between the low-molecular-weight components of subfragment 1 and those of myosin

The simplest assumption from the electrophoretic studies is that the three low-molecular-weight components observed on electrophoresis of subfragment 1 in 8 M-urea, pH 8.6, are derived from the low-molecular-weight components of the parent myosin molecule. When molecular weights were determined by gel electrophoresis of a mixture of the light components in the presence of sodium dodecyl sulphate values of 15,500, 18,500 and 23,000 were obtained for components SM1, SM2 and SM3, respectively. The first two values are the same as the molecular weights of MI and MI2/MI3 components of myosin respectively, but the SM1 component has a significantly lower molecular weight than component MI, for which a value of about 25,000 was obtained, in agreement with published values (Sarkar et al., 1971). However, recent evidence suggests that the molecular weight of component MI4 is in fact close to 22,000 (Weeds & Lowey, 1971; Perrie et al., 1972) and that it behaves anomalously in the electrophoretic system used in the present studies. Whatever its true molecular weight may be, component SM1 has a smaller apparent molecular weight than component MI in the electrophoretic system used in the present study and must be either a different protein or a product of proteolysis of component MI. The component corresponding to SM2 has a similar molecular weight to component MI of myosin and its phosphorylated form MI (Perrie et al., 1972), but is not identical with it, as judged by its electrophoretic behaviour in urea and 40% glycerol.

Further evidence which suggested similarities between components SM1 and MI of the white-skeletal-muscle myosin from which it was prepared was obtained by study of subfragment-1 preparations made from myosin isolated from rabbit cardiac muscle. Component MI1 is absent from cardiac myosin, and significantly the light-chain band corresponding to component SM1 of subfragment 1 from skeletal myosin was absent from the subfragment 1 prepared from cardiac myosin. Subfragment 1 was also prepared from the mixed skeletal muscle of myosin isolated from rabbits made dystrophic by maintaining them on a diet deficient in vitamin E. Myosin from such muscle is deficient in component M1 (Lobley et al., 1971) and likewise the subfragment 1 prepared from it also possessed less of the SM1 component as judged by the intensity of staining of the gels.
Table 3. *Amino acid composition of '26000 component' from heavy-chain fraction of subfragment 1*

For experimental details see the text. Results are expressed as mol/10^6 g of protein. —, Not determined.

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Electrophoretic patterns of subfragment 1 prepared by using different concentrations of papain

Samples of subfragment 1 in 10 mM-2-mercaptoethanol–25 mM-Tris–HCl, pH 7.6, were applied to a 40% glycerol gel prepared as described in the Materials and Methods section. (a) and (b), Subfragment 1 prepared with a myosin/papain ratio of 200:1 (by wt.); (c) and (d), Subfragment 1 prepared with a myosin/papain ratio of 2000:1. Samples (a) and (c) were stained for protein and (b) and (d) for ATPase activity.
EXPLANATION OF PLATE 2

Electrophoretic patterns of urea-treated subfragment 1 and myosin

Samples in 8M-urea–10mM-2-mercaptoethanol were applied to gels as indicated in the Materials and Methods section. Subfragment 1 was prepared with a myosin/papain ratio of (a) 200:1 or (b) 2000:1 and applied to an 8M-urea gel. (c) Myosin applied to an 8M-urea gel. Samples (d), (e) and (f), as samples (a), (b) and (c) respectively but applied to a gel containing 40% glycerol.
EXPLANATION OF PLATE 3(a)

Sodium dodecyl sulphate electrophoresis patterns of subfragment 1 preparations

Samples in 8M-urea–0.1% sodium dodecyl sulphate–10mM-sodium phosphate buffer, pH 7.0, were applied to 10% acrylamide gels. (a) Subfragment 1 prepared with a papain/myosin ratio of 1:2000; (b) subfragment 1 prepared with a papain/myosin ratio of 1:200; (c) light-chain fraction from subfragment 1; (d) myosin; (e) isolated ‘26000 component’.

EXPLANATION OF PLATE 3(b)

Electrophoretic pattern of isolated ‘26000 component’

Samples in 10M-urea–20mM-Tris–glycine buffer, pH 8.6, were applied to an 8M-urea gel. (a) Isolated ‘26000 component’; (b) subfragment 1; (c) myosin.

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Although all the low-molecular-weight components were present in the subfragment-1 preparations made from skeletal myosin with high or low concentrations of papain, there was some evidence of a lower amount of component SM12 in the preparations made with lower papain concentrations (see above) as judged by the intensity of Amido Black stain on the gel. The two enzymically active bands obtained on glycerol-containing gels of subfragment 1 prepared at a 1:200 ratio of papain were separated by cutting out the appropriate slices from the gel and laying each at the origin of another gel with 20mM-Tris–glycine buffer, pH8.6, containing 8M-urea. On subsequent electrophoresis each gave three similar light-chain bands.

Effect of papain concentration on the components of the heavy-chain fraction of subfragment-1 preparations

The similarity of the light-chain fraction of the two types of subfragment-1 preparation implied that the differences between the latter were more likely to be associated with the heavy-chain fraction. The behaviour of the two types of preparation was therefore compared on gel electrophoresis in sodium dodecyl sulphate under which conditions both the heavy- and light-chain fractions migrated (Plate 3a). Subfragment 1 prepared by low papain concentrations gave three main bands corresponding to proteins of 87000, 69000 and 26000 daltons. In addition smaller bands corresponding to molecular weights of 53000, 23000, 18500 and 15500 were present.

The three last named were identical in mobility with components identified in the separated light-chain fraction of subfragment 1. It was therefore concluded that the components of 87000, 67000, 53000 and 26000 daltons were derived from the heavy-chain fraction of subfragment 1. Sodium dodecyl sulphate electrophoresis of the heavy-chain fraction of subfragment 1, separated from the light-chain components by precipitation with aq. 66% ethanol from a 5M-guanidine–HCl solution (see Perrie & Perry, 1970), confirmed this conclusion.

As the relative amounts of the peptides derived from the heavy-chain fraction appeared from inspection of the gels to vary according to the conditions of preparation of subfragment 1, attempts were made to measure the amount of the components present by densitometric scans of the sodium dodecyl sulphate gels. The contributions of the various components, expressed in molar ratios, are summarized in Table 4.

The results although subject to unavoidable error owing to the asymmetry of the bands clearly indicate that at the higher papain concentration the ‘87000 component’ had been further degraded, presumably to give a product that dissociated in sodium dodecyl sulphate into fragments with molecular weights of 53000 and 26000. The amounts of both of the latter components in the heavy-chain fraction of subfragment 1 were much increased by high concentrations of papain. The results indicate that even if each molecule of ‘87000 component’ had been degraded into one molecule each of ‘53000’ and ‘26000 components’ it would not account for the observed increase in amounts of the ‘53000’ and ‘26000 components’. Thus, as the amount of the ‘69000 component’ was also decreased at the higher papain concentrations, the products of its further degradation must also have contributed to the increase in amounts of either the ‘53000 component’ or the ‘26000 component’ or possibly both.

Table 4. Contribution of the heavy- and light-chain fractions to the composition of preparations of subfragment 1

<table>
<thead>
<tr>
<th>Digestion conditions</th>
<th>Preparation no.</th>
<th>87000 daltons</th>
<th>69000 daltons</th>
<th>53000 daltons</th>
<th>26000 daltons</th>
<th>Total light-chain fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain (1:200)</td>
<td>1</td>
<td>0.29</td>
<td>0.62</td>
<td>1.07</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.23</td>
<td>0.60</td>
<td>0.95</td>
<td>1.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.20</td>
<td>0.60</td>
<td>0.88</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.24</td>
<td>0.61</td>
<td>0.96</td>
<td>1.23</td>
<td></td>
</tr>
<tr>
<td>Papain (1:2000)</td>
<td>1</td>
<td>0.28</td>
<td>0.52</td>
<td>0.11</td>
<td>0.68</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.28</td>
<td>0.55</td>
<td>0.16</td>
<td>0.46</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.39</td>
<td>0.48</td>
<td>0.12</td>
<td>0.40</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.32</td>
<td>0.52</td>
<td>0.13</td>
<td>0.51</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Results are expressed as mol/10^3 g. Values were obtained by dividing the percentage of the total area under densitometric scans (see the Materials and Methods section) represented by each peak by the molecular weight of the component. It is assumed that all components had equal colour response to Coomassie Blue.
Isolation and characterization of the '26000 component' from the heavy-chain fraction of subfragment 1

The electrophoretic evidence strongly suggested that the '26000 component' was derived from the heavy chain of subfragment 1. This origin was also indicated by the fact that, in contrast with the light-chain components, the '26000 component' could not be separated from the other higher-molecular-weight material by gel filtration in 7M-urea (Fig. 1). Separation could be effected, however, by gel filtration under more strongly dissociating conditions. For example, after reduction followed by carboxymethylation in 6M-guanidine-HCl of the heavy-chain fraction of subfragment 1 prepared at a ratio of papain of 1:200, the '26000 component' could be separated by gel filtration in 6M-guanidine-HCl. It could also be separated by gel filtration on Sephadex G-200 in the presence of 0.1% sodium dodecyl sulphate, after dissociation and reduction of subfragment-1 preparations by dialysis against 8M-urea-1% sodium dodecyl sulphate-20mM-sodium phosphate, pH 7.5, containing 50mM-mercaptoethanol. The procedure adopted as a routine for separation was gel filtration on Sephadex G-200 in 0.1% sodium dodecyl sulphate-20mM-sodium phosphate, pH 7.5 (Fig. 2). The fractions containing the '26000 component' together with some light-chain components were pooled and the gel filtration was repeated. When purified in this manner the '26000 component' precipitated from solution when dialysed exhaustively against 90% (v/v) acetone in water to remove as much sodium dodecyl sulphate as possible (Weber & Osborn, 1969).

Such preparations were not readily solubilized in urea alone but redissolved in 8M-urea-1% sodium dodecyl sulphate in the presence of 50mM-mercaptoethanol. The preparation obtained in this way was homogeneous on gel electrophoresis in sodium dodecyl sulphate (Plate 3a, e) and from the average of 12 estimations a molecular weight of 26000±1000 was calculated. Its amino acid composition was clearly different from that of the heaviest light-chain component of myosin, of molecular weight 25000–23000, and also from the myosin rod, but resembled that of the heavy chain of subfragment 1 (Table 3).

When isolated by procedures involving exposure to sodium dodecyl sulphate followed by its subsequent removal by acetone treatment the '26000 component' obtained was not readily resolubilized without the further addition of sodium dodecyl sulphate. To enable study in electrophoretic systems other than that containing sodium dodecyl sulphate, the '26000 component' was isolated in a more soluble form by gel filtration in the presence of 6M-guanidine hydrochloride as mentioned above. Although insoluble in water and salt solutions when isolated by this procedure the '26000 component' could be solubilized by dialysis against 10M-urea. To minimize carbamylation during dialyses the urea solutions were deionized immediately before use and mercaptoethanol (10mM final concentration) was added. This preparation migrated as two bands on electrophoresis in a 10% polyacrylamide gel containing 8M-urea and 20mM-Tris– glycine buffer, pH 8.6 (Plate 3b). Since some of the applied material did not migrate into the gel, it was not possible to decide whether the two bands represented different states of aggregation of a single component or fragments of slightly different amino acid composition.

Molecular weight of subfragment-1 preparations

Sedimentation-equilibrium studies showed that both types of subfragment-1 preparation were heterogeneous in size, for a slightly curved line was always obtained when plots of the logarithm of the fringe displacement was plotted against the square of the distance from the centre of rotation (Fig. 3).

From the maximum and minimum slopes of these plots molecular weights were obtained for both types of preparation. The range of molecular weights was 112000–133000 for subfragment 1 prepared with higher concentrations of papain and 105000–133000 for subfragment 1 prepared with lower concentrations of papain. Thus no significant differences in molecular weight could be detected between the two preparations and the heterogeneity appeared to be common to both.
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Fig. 3. Sedimentation-equilibrium studies of subfragment-1 preparations

Samples (50 μl) of subfragment-1 preparations in 0.2M-KCl – 1 mM-dithiothreitol, 25 mM-Tris - HCl buffer, pH 7.6, were loaded into the sample sector of a 12 mm capillary-type double-sector synthetic-boundary cell into which 20 μl of FC 43 (a fluorocarbon oil supplied by Beckman RIIC Ltd., used to provide a base for the solution column) had previously been introduced. The reference sector contained 20 μl of FC 43 and 0.42 ml of solvent. Samples were centrifuged at 14290 rev./min at 4°C until equilibrium was attained (8-10h). (a) Subfragment 1 prepared at a myosin/papain ratio of 200:1, initial concn. 0.75 mg/ml; (b) subfragment 1 prepared at a myosin/papain ratio of 2000:1, initial concn. 0.63 mg/ml.

Discussion

The heterogeneity of subfragment 1 prepared at high papain concentration that is observed on electrophoresis in non-dissociating conditions is largely one of charge and a consequence of extensive digestion in the heavy-chain fraction of subfragment 1. The increased proteolysis neither removed much additional peptide material nor modified the enzymic centre, since the molecular weights and enzyme activities of both preparations were not significantly different. The sedimentation results indicate that in both preparations there was some heterogeneity of molecular weight. In view of the findings it is considered that a papain/myosin ratio not exceeding 1:1000 should be used to obtain subfragment-1 preparations that are electrophoretically homogeneous. Standard procedures for subfragment-1 preparation currently in use employ a range of ratios of proteolytic enzyme to myosin, and some methods would be expected to produce preparations that contain two enzymically active components.

The differences observed between the light-chain fractions of the two types of subfragment-1 preparation are small and are probably unlikely to be the cause of the differences in electrophoretic behaviour of the undissociated preparations. For given preparative conditions the electrophoretic pattern of the light-chain fraction of subfragment 1 was very consistent in contrast to the somewhat variable patterns reported by Weeds & Lowey (1971), who noted that the subfragment 1 contained little of the 5,5-dithiobis-(2-nitrobenzoic acid) light chain. The light-chain component of myosin of highest electrophoretic mobility, M11 (molecular weight 15000), is probably little changed during formation of subfragment 1, but the light-chain component of myosin of lowest mobility, M14 (molecular weight 23000-25000), is modified. It cannot be decided from this study whether component M14 is the precursor of either or both of the two electrophoretically slower light components of subfragment 1, SM14 and SM13. It is possible that the two last-named components were derived from the heavy-chain fraction, but this seems unlikely in view of the amino acid analysis and their solubility at low ionic strength. The observation that the SM13 band is more intense in subfragment-1 preparations made with higher papain concentrations suggests that it is derived from another component of higher molecular weight, rather than the light-chain component of myosin (M12) of molecular weight 18000. The fact that proteolytic modification of the so-called alkali light chains of myosin accompanies subfragment-1 formation, both from skeletal and cardiac muscle, implies that they are not required in the precise form in which they are present in myosin for the preservation of enzymic activity.

The heavy-chain fraction of the subfragment 1 prepared with the higher papain concentration is clearly more extensively degraded than that obtained with the lower papain concentration. The two components of subfragment-1 preparations observed on electrophoresis of the native protein have not been
separated and studied; nevertheless the evidence suggests that the component that is restricted to the preparations obtained with the highest papain concentration contains more of the lower-molecular-weight fragments of the heavy chain.

The '26000 component' probably represents a unique sequence that is not repeated along the heavy chain. This conclusion is supported by the yield, which approaches 1 mol/mol of subfragment 1 under conditions of high papain concentration. Also, all the mono-$N^\alpha$-methyl-lysine and part of the tri-$N^\alpha$-methyl-lysine and 3-methylhistidine are found in the '26000 component'. The amount of mono-$N^\alpha$-methyl-lysine present amounts to about half a residue per molecule, as is the case with subfragment 1. This implies that only half of the molecules in a preparation of subfragment 1, and the '26000 component' derived from it, contain mono-$N^\alpha$-methyl-lysine. Nevertheless, although the analytical results and the electrophoretic studies suggest heterogeneity in composition of the '26000 component', it presumably reflects the heterogeneity that was present in the original subfragment 1 and the myosin from which it was derived.

If the '26000 fragment' represents a unique sequence of the heavy chain some conclusion as to its origin can be made from study of the yields estimated from densitometric scanning of the sodium dodecyl sulphate gels. The results suggest that the further proteolysis of the '87000 component' that occurs at higher papain concentrations produces a fragment which dissociates in the presence of sodium dodecyl sulphate into components of 53000 and 26000 daltons. This would not account for all of the '26000 component' present, however, since all preparations contain more molecules of the '26000 component' than of the '53000 component'. Although further proteolysis of the '69000 component' could theoretically yield the '26000 component', this seems unlikely, since no fragments of around 40 000 molecular weight are apparent in the preparations. It seems possible that the remainder of the '26000 component' is derived from a longer chain of which the '69000 component' also formed a part in the native preparations. This conclusion is consistent with the densitometric-scanning results, which indicate that the number of molecules of '26000 component' is approximately equal to the total number of molecules of '69000 component' and '53000 component'.

The present study has shown that subfragment-1 preparations are enzymically active despite the fact that the polypeptide chain derived from the myosin heavy chains is not intact. The electrophoretic results imply that in most of the molecules in all subfragment-1 preparations there is at least one break in the heavy chains. The fragments must be held together by side-chain interactions, as the molecular weights in non-dissociating conditions are relatively unchanged despite the increase in the number of bonds broken in the heavy chain of subfragment 1 produced by high papain concentration. In the latter case, in 60% of the molecules the largest piece of heavy chain has a molecular weight of only 53000 without apparent loss in enzymic activity.

It cannot be concluded from the information available that the heterogeneity observed in subfragment 1 prepared at higher papain concentrations reflects major differences in the structure of the two heads of the myosin molecule, although minor structural differences may be present in the part of the myosin molecule that gives rise to subfragment 1. It is more likely that the heterogeneity observed reflects the presence of bonds of different susceptibility in common to all the myosin molecules, and which are hydrolysed at a significant rate in the presence of higher papain concentrations.

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References

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