FRACTIONATION OF HEAVY MEROMYOSIN BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Fast-twitch muscle myosin contains two heavy chains and two pairs of light chains, one class of which can be largely removed without loss of ATPase activity [1]. These 19 000 mol. wt light chains (often termed DTNB light chains) may be involved in divalent cation binding to the thick filaments [2–4] and possibly influence actin interaction [5]. The other pair of light chains are heterogeneous and have been termed Alkali 1 (A1 27 000 mol. wt) and Alkali 2 (A2 16 500 mol. wt) respectively. They cannot be removed without loss of enzymatic activity indicating that they are tightly associated with the heavy chains. Although their amino acid sequences are identical over most of their length, they are genotypically distinct [6]. Furthermore, they do not occur in equal or integral yields indicating the presence of isoenzymes [7]. In adult rabbit myosin the molar ratio of A1 : A2 is about 2, while in chicken myosin it is closer to unity [8,9].

Fractionation of rabbit subfragment-1 (S-1) into S-1 (A1) and S-1 (A2), isoenzymes separated on the basis of the different light chains, has been achieved using ion-exchange chromatography [10,11] or affinity chromatography [12]. The ATPase activities of these isoenzymes are indistinguishable in the absence of actin [11,13] but the actin-activated ATPase of S-1 (A2) has a higher maximal velocity (k_{cat}) and a larger K_m (the apparent dissociation constant for actin in the presence of ATP) than S-1 (A1) [11,14]. Recent experiments have shown that these differences are due to the alkali light chain present. The light and heavy chains may be reversibly dissociated and, following recombination, the influence of the particular Alkali light chain on the actin-activated ATPase can be clearly demonstrated [14]. It is important to establish that these results are not artefacts arising from using S-1, a single-headed proteolytic fragment of myosin, but apply equally to two-headed species. Therefore heavy meromyosin (HHM) has been fractionated according to its light chain content using an ADP affinity column. The actin-activated ATPase of HMM was found to be dependent on the particular alkali light chain in a manner similar to that observed for S-1.

2. Materials and methods

Fast-twitch muscle myosin was isolated from either rabbit back- and hind-leg muscles or from chicken breast muscle [15]. S-1 was prepared from these myosins by chymotryptic digestion in 0.12 M NaCl, 20 mM sodium phosphate (pH 7.0), 1 mM EDTA and 1 mM dithiothreitol as described previously [14]. HMM was also prepared by chymotryptic digestion of these myosins in a solution containing 0.6 M NaCl, 20 mM sodium phosphate (pH 7.0) and 1 mM $CaCl_2$ [4]. To minimize degradation of the DTNB light chains in these HMM preparations, digestion was stopped after 2 min by addition of phenyl methane sulphonyl fluoride (100 mM in isopropyl alcohol) to a final concentration of 0.5 mM. Concentrations of S-1 and HMM were estimated by absorbance using values for $A^{1\%}_{280}$ of 7.5 cm^{-1} and 6.5 cm^{-1}, respectively [4]. Molecular weights of 115 000 and 350 000 were used for S-1 and HMM, respectively.

Actin was prepared from acetone powders of both rabbit muscle and chicken muscle and concentrations estimated using an absorbance value $A^{1\%}_{280}$ of 11.0 [16]. These two actin preparations were indistinguishable in their effects on the actin-activated ATPase activities of S-1 or HMM.

Sepharose 4B adipic acid hydrazide–ATP was prepared as described by Lamed et al. [17] and the ADP analogue synthesized in the same manner. Although the ADP affinity columns could be re-used several times, the concentration of ADP or pyrophosphate required to elute the different fragments decreased and there was a concomitant decrease in resolution of the columns. Variation in binding capacity between different preparations of the affinity matrix were also noted.

Polyacrylamide gel electrophoresis (using 10% acrylamide) in the presence of sodium dodecyl sulphate was used to determine the light chain composition of the different fragments and was carried out as described previously [4]. The staining intensities of the different bands were determined by densitometry and stoichiometries calculated using the appropriate molecular weights. Each protein sample was run at four different concentrations and average molar ratios determined.

ATPase activities were measured as described previously [14]. A pH-stat was used for all ATPase assays except for the Mg^{2+}-dependent ATPase for which a spectrophotometric linked assay system was used. Actin activated ATPase activities were assayed using varying concentrations of actin, at pH 8.0 and 25°C. Values for k_{cat} and K_m were calculated by extrapolation from Eadie-Hofstee plots.

3. Results and discussion

Attempts to fractionate S-1 with Sepharose 4B adipic acid hydrazide–ATP as described by Yoshida and Morita [12] were unsuccessful. However, by using the ADP derivative instead, satisfactory separations of both rabbit and chicken S-1 isoenzymes were obtained. The ADP affinity column was equilibrated in 10 mM Tris–HCl (pH 7.6, 4°C), 40 mM NaCl, 1 mM EDTA and 0.1 mM dithiothreitol and S-1 fractionated using gradients of either 0.0–6.0 mM ADP or 0.0–10.0 mM sodium pyrophosphate in the same buffer. Two well resolved fractions were obtained with S-1 (A2) eluting first. The main advantage of this method over ion exchange is that S-1 (A2) was less contaminated by S-1 (A1) and was obtained in higher yields.

A major difficulty in analyzing the extent of fractionation of HMM arises from degradation of the DTNB light chains [4]. Digestion of myosin by chymotrypsin in the presence of divalent cations results in a fragment of the DTNB light chains which co-migrates with the A2 light chain on gel electrophoresis (both in sodium dodecyl sulphate and in urea-containing gels). The extent of this degradation can be minimised by limiting the time of digestion, but even with a 2 min digest of chicken myosin the ratio observed for the three light chains charges from 1:2:1 to about 1:1.5:1.5 (A1:DTNB:A2), indicating degradation of about 25% of the available DTNB light chains.

Fractionation of chicken HMM on the ADP affinity column is shown in fig.1. The breakthrough peak contained a number of unidentified digestion products and the HMM eluted in three fractions. The first of these peaks was high in A2 and very low in A1, the second peak contained high levels of both A1 and A2 and the third peak was substantially enriched in A1. Analysis of pooled fractions by gel electrophoresis (fig.2) was used to determine the relative light chain contents. Table 1 shows the observed molar ratios for the three light chains in the different fractions and values corrected for the degradation of the DTNB light chain. This correction is only approximate since it assumes that no further degradation of the DTNB light chains occurs and that there are no other contaminants co-migrating with these bands on gel electrophoresis. Further it assumes that there is no preferential fractionation of the

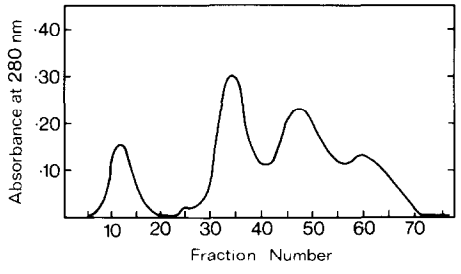

Fig.1. Fractionation of chicken HMM with a Sepharose 4B adipic acid hydrazide–ADP column. The column (1.5 × 30 cm) was equilibrated in 10 mM Tris–HCl, pH 7.6 at 4°C, 1 mM EDTA and 0.1 mM dithiothreitol. The HMM (150 mg) was loaded and washed with this buffer. Starting at fraction 17, a 1–10 mM sodium pyrophosphate gradient, 400 ml each, was applied.

DTNB light chains or its degradation products on the affinity column. While HMM (A2) is obtained with little contamination by A1, the A1-enriched fraction contains significant amounts of A2 even after the appropriate correction has been applied. The amount of DTBN light chain in the A1-enriched HMM is less than in the other fractions which may imply that degradation is more extensive in this material. Clearly the presence of this additional 17 000 mol. wt component complicates interpretation of the light chain content. Nevertheless the enrichment that has been achieved is sufficient to allow for an examination of the influence of the alkali light chains on the actin activated ATPase.

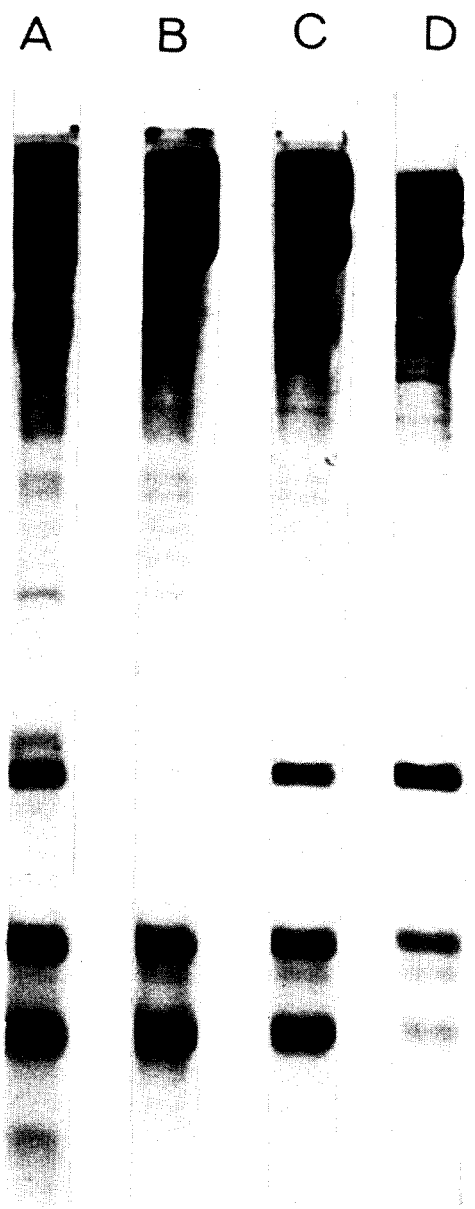

Fig.2. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. (A) Unfractionated chicken HMM, (B) fractions 30–38, fig.1, (C) fractions 42–52, fig.1 and (D) fractions 58–66, fig.1.

The ATPase activities of the three fractions in the absence of actin were similar. Values obtained for the Ca^{2+}-dependent ATPase were 7 s^{-1}; for the EDTA ATPase, 11 s^{-1} and for the Mg^{2+}-dependent ATPase, 0.024 s^{-1} (assuming both ATPase sites to be independently active). ATPase activities of the A2-enriched HMM were about 10% higher than the A1-enriched protein possibly due to inactive HMM in the latter fraction.

Significant differences were observed in the actin activated ATPase activities of the three fractions (fig.3). Both k_{cat} and K_m for the A2-enriched HMM are much higher than those for the A1-enriched material (Table 2), while intermediate values were found for the middle fraction. Occasionally non-linear Eadie-Hofstee plots were obtained with protein from the middle fraction, possibly indicating the presence of kinetically heterogeneous species.

When the middle fraction was reapplied to the ADP affinity column, it eluted as a single peak in the same position on the gradient, indicating a single species rather than a mixture of the two enriched components. A mixture of the enriched HMM species was refractionated on the affinity column and shown to separate into two distinct peaks. One interpretation of these observations is the presence of heterodimers, HMM species containing both A1 and A2 light chains, but the elution position of a particular HMM species may also reflect the state of the DTNB light chain or differences in the heavy chains. Thus it is premature to conclude the presence of heterodimers from these experiments. Indeed, Holt and Lowey [18] have concluded that chicken myosin is composed predominantly of homodimers.

Rabbit HMM was also fractionated on the ADP affinity column under similar conditions and enrichment of the alkali light chains observed. ATPase

Table 1
Light chain ratios of fractionated chicken HMM

	Unfractionated		A2-Enriched peak		Middle peak		A1-Enriched peak	
	I	II	I	II	I	II	I	II
A1	1.00	1.00	0.02	0.02	1.00	1.00	1.00	1.00
DTNB	1.56	2.00	0.61	0.78	1.60	2.05	0.62	0.79
A2	1.59	1.15	1.00	0.83	1.80	1.35	0.42	0.25
A1/A2	0.63	0.87	0.02	0.025	0.56	0.74	2.38	4.00

The observed molar ratios (I) are averages from three separate HMM fractionations. The adjusted ratios in II are calculated assuming 22% degradation of the DTNB light chain to a 17 000 mol. wt fragment. The amount of observed DTNB light chain was multiplied by 0.28. This value was added to the observed DTNB light chain and subtracted from the observed A2 light chain. With the exception of the A2 enriched peak, observed ratios are normalized to A1

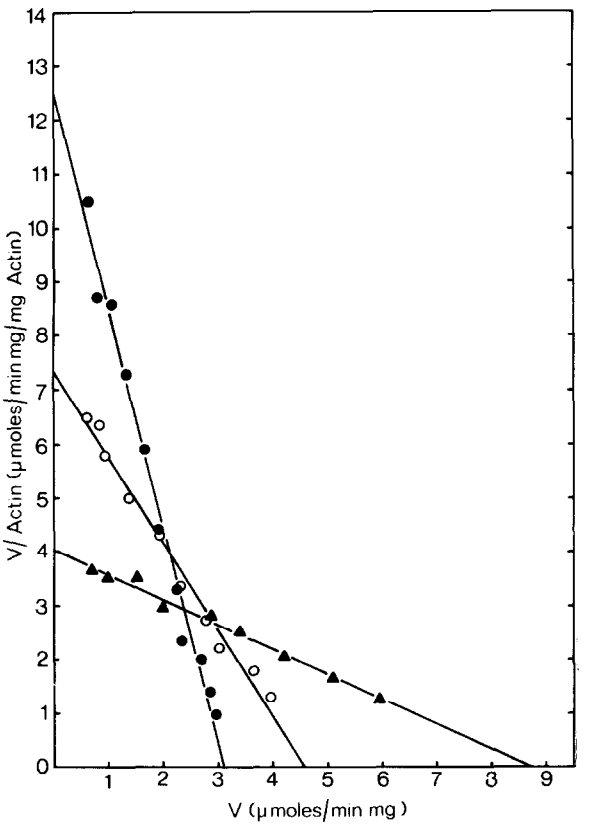

Fig.3. Eadie-Hofstee plots of actin activated ATPases of chicken HMM. (•) HMM enriched in A1, (○) mixed HMM, middle peak and (▲) HMM enriched in A2.

activities in the presence of actin were consistent with results obtained above.

Previous experiments have shown that the alkali light chains influence the interaction of S-1 with F-actin [11,14]. When the A2 light chain is bound to S-1, the actin activated ATPase has a higher k_{cat} and a larger K_m than when the A1 light chain is bound. The work presented here demonstrates that the alkali light chains have the same effect on HMM as they do on S-1. HMM which is enriched in A2 has an actin activated ATPase like two independent S-1 (A2)s and HMM enriched in A1 is like two S-1 (A1)s, table 2. The fractionation of HMM by affinity chromatography on Sepharose 4B adipic acid hydrazide–ADP columns shows that homodimers of myosin do exist. However, the question of heterodimers cannot be resolved from these experiments.

Table 2
Actin-activated ATPase activities of chicken HMM and S-1 species

	k_{cat} (s^{-1})	K_m (μM actin)
A2-Enriched HMM	30.0 (3.9)	57.2 (5.7)
Middle fraction	17.8 (3.9)	16.8 (1.8)
A1-Enriched HMM	10.6 (2.2)	5.4 (1.5)
S-1 (A2)	34.1	53.2
S-1 (A1)	14.7	6.4

Values of k_{cat} and K_m for the HMM species are averages from three separate preparations. The numbers in parentheses are standard deviations. Values for the S-1 isoenzymes are the mean of two determinations. k_{cat} are per active site

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