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Distribution of Alkali Light Chains in Myosin: Isolation of Isoenzymes†

John C. Holt‡ and Susan Lowey*

ABSTRACT: Antibodies have been isolated which are specific for the "difference peptide" unique to the alkali 1 light chain (mol wt 20 700) of chicken breast muscle myosin. When coupled to Sepharose as an immunoadsorbent, they are capable of resolving subfragment 1, heavy meromyosin, and myosin into two fractions, one rich in alkali 1 and the other rich in alkali 2. This fractionation provides direct evidence for the existence of two isoenzymic populations in vertebrate skeletal myosin. The ability of antibodies to the difference peptide to distinguish between alkali 1 and 2 provides a marker which will allow the distribution of alkali light chains in muscle fibers and filaments to be investigated.

The low molecular weight subunits of vertebrate skeletal myosin fall into two classes: DTNB light chains (2 mol/mol of myosin), which can be selectively dissociated by the thiol reagent DTNB,[1] and alkali 1 and 2 light chains (a total of 2 mol/mol of myosin), which are released only under denaturing conditions such as exposure to pH 11 (Weeds, 1969; Gazith et al., 1970; Weeds and Lowey, 1971). The two species of alkali light chains are closely related, although clearly the products of different genes (Weeds and Frank, 1972). The sequence of alkali 2 (mol wt 16 500) is repeated in alkali 1 (mol wt 20 700) with only five substitutions. The unique feature of alkali 1 is an N-terminal sequence of 41 residues which accounts for its higher molecular weight (Frank and Weeds, 1974). This sequence, which is rich in proline, alanine, and lysine, is termed the "difference peptide".

There are two distinct ways in which the closely related alkali 1 and 2 subunits may be distributed in myosin. Either a single type of myosin molecule exists, in which the two heads differ with respect to their alkali l.c. (heterodimers) or, alternatively, there may be two isoenzymic forms of myosin with the two heads of each molecule containing the same light chain (homodimers). A third possibility which must be recognized is the occurrence of a mixture of homodimers and heterodimers.

The isoenzyme interpretation has received some indirect support from observations on the stoichiometry of alkali l.c. in rabbit skeletal myosin. The light chains have been found to occur in the proportions 1.2-1.4 mol of alkali 1 to 2 mol of DTNB l.c. to 0.6-0.8 mol of alkali 2 (Lowey and Risby, 1971; Sarkar, 1972; Weeds et al., 1975). The unequal amounts of alkali 1 and 2 have been taken to imply that the myosin contains unequal amounts of two homodimers, rather than a uniform population of heterodimers. Since both alkali l.c. were found not only in mixed back and leg muscles but also in single fibers taken from a more histochemically homogeneous region of the psoas (Weeds et al., 1975), the isoenzymes cannot arise simply from the mixing of different fiber types. It must be pointed out, however, that in myosin from chicken breast muscle the amounts of alkali 1 and 2 are essentially equal (Lowey and Risby, 1971), so that the existence of homodimers is not necessarily to be inferred.

A more direct approach to the distribution of the alkali l.c. in a myosin preparation is to fractionate the presumed isoenzymes. So far this has been achieved for S1, the single head of myosin, which can be resolved into alkali 1 and 2 types by chromatography on DEAE-cellulose (Yagi and Otani, 1974; Weeds and Taylor, 1975). Information on the composition of intact myosin must come, however, from the fractionation of

† From the Rosenstiel Basic Medical Sciences Research Center and the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received March 28, 1977.* This work was supported by grants from the National Science Foundation (PCM 75-14790), the National Institute of Arthritis, Metabolism and Digestive Diseases, U.S. Public Health Service (AM 17350), and the Muscular Dystrophy Association, Inc.

‡ Present address: National Institute for Biological Standards and Control, London NW3 6RB, U.K.

[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HMM, heavy meromyosin; S1, subfragment 1; EDTA, ethylenediaminetetraacetic acid; Gdn·HCl, guanidine hydrochloride; DEAE, diethylaminoethyl.

a double-headed species, i.e., HMM or myosin. We describe here an immunochemical method which can be used to fractionate myosin and its subfragments into alkali 1 and 2 isoenzymes. Antibodies to the difference peptide have been isolated from antiserum to alkali 1 and coupled to Sepharose to form an immunoadsorbent specific for alkali 1. When S1, HMM, or myosin was passed through columns of this material, a fraction rich in alkali 2 was eluted without retardation, while a second fraction, rich in alkali 1, was retained on the column and could be eluted with guanidine hydrochloride. The fractionation of HMM and myosin into two populations provides for the first time direct evidence that there exist at least two types of vertebrate skeletal myosin.

Materials and Methods

Preparation of Proteins. Chicken breast muscle myosin was prepared as described by Holtzer and Lowey (1959).

Tryptic HMM and papain S1 were isolated as previously described (Holt and Lowey, 1975b), except that digestion of insoluble myosin with papain was carried out in the presence of 2 mM $MgCl_2$ to protect the DTNB l.c. from degradation (Margossian et al., 1975). The water-soluble fraction of the digest was purified by chromatography on DEAE-cellulose at pH 7.9 (Lowey et al., 1969). Papain S1 was resolved into alkali 1 and 2 types by chromatography on DEAE-Sephadex A-50 equilibrated in 0.05 M imidazole·HCl, pH 7, 0.06 M NaCl; fractions were eluted with a gradient of 0–0.12 M NaCl in the same solvent (Weeds and Taylor, 1975). Chymotryptic subfragments were obtained essentially as reported by Weeds and Taylor (1975) for rabbit myosin. Our only modification in digesting chicken myosin was to use less chymotrypsin (0.025 mg/mL) and a shorter incubation time (5 min). The subfragments were purified by fractionation between 43% and 58% saturated $(NH_4)_2SO_4$.

The preparation of purified light chains, and antisera to them, was carried out as described earlier (Holt and Lowey, 1975a).

Preparation of Antibody to the Difference Peptide. The principle used was to absorb all antibodies cross-reacting with alkali 2 from anti-alkali 1 serum, so that only antibodies reactive with the difference peptide remained. These comprised 5–40% of the total anti-alkali 1 antibody in different sera. Experimentally, anti-alkali 1 serum was passed through several columns of Sepharose-coupled alkali 2 (see below). The desired antibody was that which passed unretarded through each column. The progress of the antibody fractionation was followed by eluting the columns at pH 2 or with 4 M Gdn·HCl to determine how much anti-alkali 1 had been bound. In a typical preparation, 165 mL of serum containing 195 mg of precipitable anti-alkali 1 was passed through three columns, each containing about 30 mg of alkali 2 coupled to 25 mL of Sepharose 4B. The first column bound 120 mg, the second 30 mg, and the third, less than 2 mg of anti-alkali 1 antibody. Finally the antibody remaining in the serum was adsorbed on a column of Sepharose-coupled alkali 1; the yield after elution at pH 2 and rapid neutralization was 34 mg. The close agreement between the total precipitable anti-alkali 1 and the sum of the bound anti-alkali 1 and the difference antibody must be regarded as fortuitous, since nonprecipitating antibody is not accounted for in the precipitin reaction. When anti-alkali 1 was absorbed with alkali 2 at just less than equivalence (based on a quantitative precipitin test), appreciable amounts of anti-alkali 1 remained in the serum after removal of the antigen–antibody precipitate.

Preparation of Immunoadsorbents. Purified light chains were coupled in 0.1 M $NaHCO_3$, pH 9, to Sepharose 4B activated with 30 mg of CNBr/mL packed gel following the procedure of Omenn et al. (1970). After 20 h at 4 °C with gentle stirring, coupling was assumed to be complete since no free protein could be washed from the gel. On the basis of this assumption the concentration of bound protein was 1.5 mg/mL packed gel. Difference antibody was coupled in 0.15 M KCl, 0.02 M potassium phosphate, pH 7.2, to Sepharose 2B activated with 200 mg of CNBr/mL of packed gel. The concentration of bound protein was about 1 mg/mL of packed gel. Since coupling is less efficient at the lower pH, more CNBr was used to provide more reactive groups on the matrix. The advantage of the lower pH is that fewer uncharged lysine residues on the protein are available for attachment to the matrix, and the coupled protein may, therefore, be more reactive (Porath and Kristiansen, 1975).

After coupling, all immunoadsorbents were washed for 1 h with 0.5 M ethanolamine, pH 9, and stored at 4 °C in 0.02% NaN_3 and a trace of toluene. They remained functional for several months under these conditions.

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis on 9, 10, or 12% polyacrylamide gels containing 0.1% dodecyl sulfate was carried out as described previously (Holt and Lowey, 1975a). Samples eluted from immunoadsorbent columns with 4 M Gdn·HCl were diluted twofold with water and dialyzed for 2–4 h against 10 mM sodium phosphate, pH 7. They were then treated for 2 min at 100 °C with 1% dodecyl sulfate, 1 mM dithiothreitol. This procedure was followed in order to minimize proteolytic degradation of S1 at intermediate concentrations of denaturant.

Gels were stained and destained by diffusion (Fairbanks et al., 1971) and scanned on a Joyce-Loebl microdensitometer. Dye-binding was taken to be the same for all light chains (unpublished observations).

Fractionation Experiments. Immunoadsorbent columns of 3 mL volume (3 mg of bound antibody) were equilibrated in 0.15 M KCl, 0.02 M potassium phosphate, pH 7.2, at a flow rate of 14 mL/h. Although most experiments were carried out at 4 °C, the fractionation of tryptic HMM was equally good at 25 °C. Samples containing 1 mg of myosin or subfragment were applied in a volume of 0.2 mL. The columns were monitored at 230 nm using a Beckman DB-GT spectrophotometer equipped with a flow cell. When the recorder trace returned to the baseline after elution of the unretained material, a step of 4 M Gdn·HCl (pH 7.2 in the column buffer) was applied to release the bound fraction. A complete experiment took ~2.5 h. Recovery was 80–90% of the protein applied, based on manual measurements of the extinction of 0.8-mL fractions in two experiments. Samples for gel electrophoresis were usually taken as peak fractions and represented about 50% of the protein in the whole peak.

Results

Antisera to alkali 1 and 2 show considerable cross-reactivity and so cannot themselves be used to distinguish between these light chains (Holt and Lowey, 1975a). Anti-alkali 1, however, contains antibodies specific for the difference peptide which is present uniquely in alkali 1 (Frank and Weeds, 1974). These "difference antibodies" were isolated by making use of their inability to react with alkali 2. The specificity of the antibody preparation was shown by a weak immunodiffusion reaction with alkali 1, but none with alkali 2. As a more critical demonstration, binding of ^{125}I-labeled light chains to the difference antibody occurred ~4000× more readily with alkali 1 than with alkali 2 (L. Silberstein, personal communication).

Immunoadsorbent columns of difference antibody were capable of fractionating chymotryptic S1 and tryptic HMM

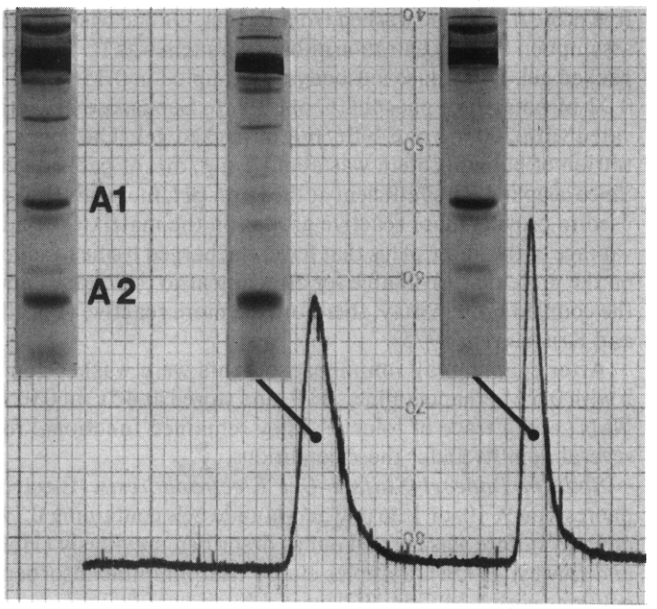

FIGURE 1: Elution profile of chymotryptic S1. Details are given in Materials and Methods. The dodecyl sulfate gels (9% polyacrylamide) show from left to right: the starting material; the unretained alkali 2 fraction; and the 4 M Gdn·HCl-eluted alkali 1 fraction.

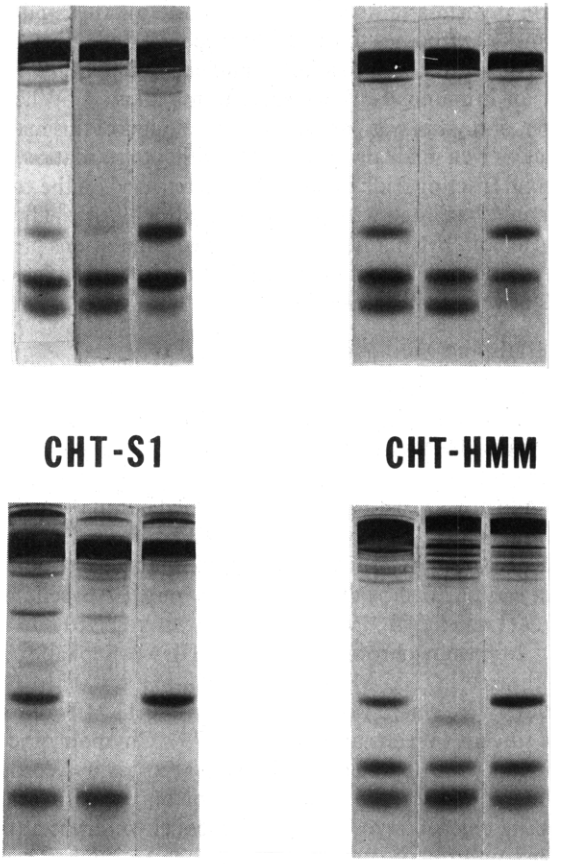

FIGURE 2: Dodecyl sulfate gel electrophoresis of S1 and HMM. In each group, the left-hand gel shows the starting material, the middle gel, the alkali 2 fraction, and the right-hand gel, the alkali 1 fraction. Papain S1 was fractionated both on DEAE-cellulose at pH 7 (gels on left), and on a difference antibody column (gels on right). Chymotryptic (CHT−) subfragments were fractionated immunochemically.

almost entirely into alkali 1 and 2 types (see below). We were therefore puzzled by the observation that myosin, chymotryptic HMM, and papain S1 were largely bound by the antibody column, without discrimination between alkali l.c. A common feature of the subfragments that were successfully fractionated is the degradation of their DTNB l.c. during the proteolytic cleavage of myosin. Chymotryptic S1 contains no detectable DTNB l.c., while this light chain in tryptic HMM has a molecular weight of about 17 500, compared with 18 500 for the intact molecule (see the gels in Figures 2 and 3). Immunological cross-reactivity has, moreover, been observed between alkali 1 and DTNB l.c. (Silberstein and Lowey, 1977). It therefore appeared possible that the binding of myosin and those subfragments with less-degraded DTNB l.c. to the immunoadsorbent might be through this light chain. This hypothesis was proved correct when prior absorption of the difference antibody with DTNB l.c. produced a marked improvement in the fractionation. Once the cross-reactivity with DTNB l.c. had been established, difference antibody was routinely passed through Sepharose-coupled DTNB l.c. before use.

A typical elution profile for chymotryptic S1 is shown in Figure 1, together with dodecyl sulfate gels of the starting material and the two fractions obtained from the column. The first fraction consisted of S1 which passed unretarded through the column, and contained only alkali 2. The second fraction remained bound while the column was washed, and was eluted only with 4 M Gdn·HCl. Neither 8 M urea nor pH 2 was effective as an eluting agent. The gels show that the fraction which bound to the immunoadsorbent contained only alkali 1. The fractionation of chymotryptic S1 by immunochemical methods therefore parallels that obtained by ion-exchange chromatography (Yagi and Otani, 1974; Weeds and Taylor, 1975). When prepared in the presence of Mg^{2+}, papain S1, unlike chymotryptic S1, contains a full complement of DTNB l.c. (Margossian et al., 1975). Despite this difference, papain S1 could also be fractionated by either ion-exchange or affinity chromatography (Figure 2). The absence of DTNB l.c. is not, therefore, a prerequisite for the separation of alkali 1 and 2 isoenzymes, except in relation to the immunological cross-reactivity mentioned above.

Although these experiments established the specificity of the columns with respect to the binding of alkali 1 and 2, only the fractionation of a two-headed species can provide information on how alkali 1 and 2 are distributed in myosin. The elution profile obtained when tryptic HMM was applied to a difference antibody column is shown in Figure 3, together with dodecyl sulfate gels of the starting material and the fractions obtained from the column. That the HMM has been resolved into two components, one rich in alkali 1, the other rich in alkali 2, is confirmed by the densitometer traces (from an independent experiment) in Figure 4. The stoichiometry of the light chains (legend to Figure 4) is somewhat uncertain due to the proximity of DTNB l.c. and alkali 2 on the gels, and also due to the possibility that some DTNB l.c. has been even further cleaved so that it comigrates with alkali 2. It is clear, nevertheless, that the two fractions are significantly different in their contents of alkali 1 and 2, relative to DTNB l.c. No enhancement of the fractionation was observed when the amount of subfragment applied to the column was decreased threefold. The capacity of the column had not therefore been exceeded under the normal conditions of loading.

Chymotryptic HMM could also be fractionated (Figure 2) but, in this case, some DTNB l.c. is known to comigrate with

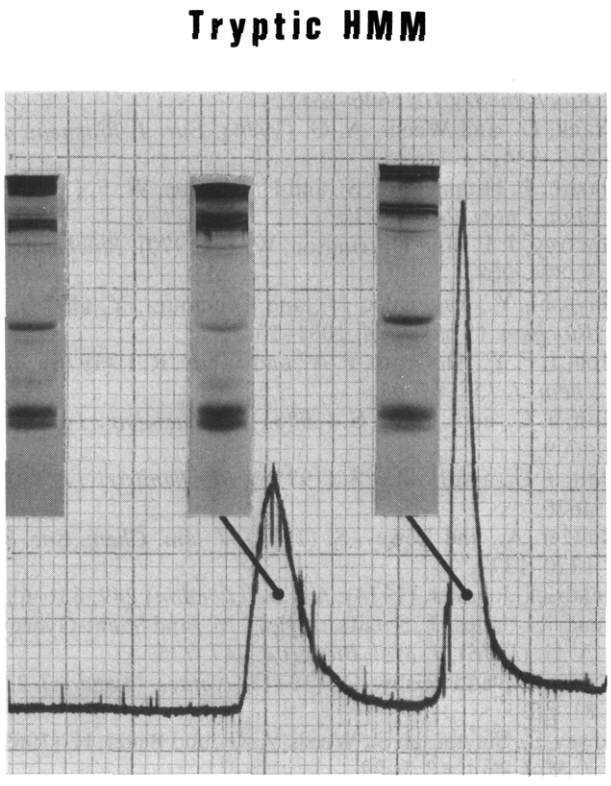

FIGURE 3: Elution profile of tryptic HMM. Conditions were as in Figure 1 except that the concentration of acrylamide in the gels was 12%.

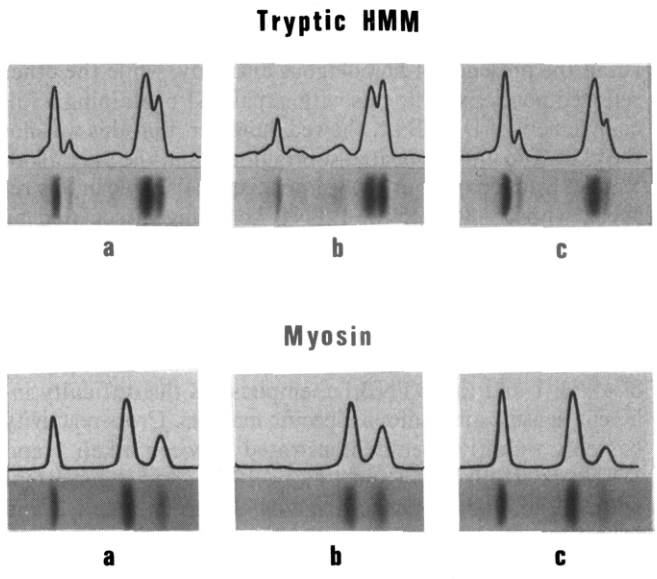

FIGURE 4: Densitometry of HMM and myosin before and after fractionation. (a) Starting material; (b) alkali 2 fraction; (c) alkali 1 fraction. The light chain compositions of the different species, normalized to 2 mol of DTNB l.c., were as follows. HMM (mean of six experiments): (a) 1.07 mol of alkali 1 to 0.95 mol of alkali 2; (b) 0.47 mol of alkali 1 to 1.48 mol of alkali 2; (c) 1.59 mol of alkali 1 to 0.42 mol of alkali 2. Myosin (mean of two experiments): (a) 0.94 mol of alkali 1 to 0.96 mol of alkali 2; (b) no detectable alkali 1, 1.64 mol of alkali 2; (c) 1.21 mol of alkali 1 to 0.65 mol of alkali 2. Molecular weights used were: alkali 1, 20 700; DTNB l.c., 18 500 in myosin, 17 500 in HMM; alkali 2, 16 500.

alkali 2 (Weeds and Pope, 1977). Thus the alkali 1 (bound) fraction will always have a component with the electrophoretic mobility of alkali 2. The alkali 2 (unbound) fraction, however, contains no alkali 1 and only a trace of a component with a slightly lower apparent molecular weight, which might derive from alkali 1. Myosin itself is free of the ambiguities caused by the use of proteolytic enzymes in the preparation of the subfragments, but is generally more difficult to handle in solution. Figure 4 (lower part) shows the results which were obtained with myosin. The alkali 2 fraction is free of alkali 1, but the alkali 1 fraction contains a significant amount of alkali 2. The light chain compositions are given in the legend to Figure 4. The appearance of alkali 2 in the alkali 1 fraction may simply reflect the presence of small aggregates which might be expected in a 3 mg/mL solution of myosin (Godfrey and Harrington, 1970). This was the lowest concentration at which myosin could be applied to the immunoadsorbent if the unretarded fraction was to be detected after dilution on the column.

Discussion

The fractionation of S1 into alkali 1 and 2 isoenzymes can be carried out either by ion-exchange chromatography (Yagi and Otani, 1974; Weeds and Taylor, 1975) or by affinity methods as described here. Only with double-headed species (HMM or myosin), however, is it possible to reach any conclusion about the distribution of alkali l.c. in myosin. The isolation of two fractions, one rich in alkali 1 and the other rich in alkali 2, provides in this case strong evidence for the existence of alkali light chain isoenzymes in myosin. Although there is some uncertainty in the densitometry of HMM samples, these results, together with the more limited data on myosin, point to the same conclusion.

The incomplete fractionation of myosin may arise through nonspecific binding of denatured or aggregated protein to the immunoadsorbent; or, alternatively, there may exist a population of heterodimers in addition to the homodimers. These would presumably bind by virtue of their alkali 1 content and thus appear in the alkali 1 fraction. A possibility which seems less likely is that a uniform population of heterodimers is partially converted to homodimers by light chain exchange in the presence of the antibodies on the immunoadsorbent column. Release of alkali l.c. from myosin requires strong dissociating conditions such as exposure to pH 11 or 4.7 M NH_4Cl, unlike DTNB l.c. which appears much less strongly bound. While homologous antibody is capable of dissociating DTNB l.c., no evidence of alkali l.c. dissociation was found in parallel experiments (Holt and Lowey, 1975b). Elution of the alkali 2 fraction is, moreover, complete in 45 min, compared with 10–50 h for DTNB l.c. dissociation by antibody (Holt and Lowey, 1975b).

Different electrophoretic forms of myosin have been demonstrated both in different muscles, e.g., fast and slow, and more recently, even in the same muscles (Hoh et al., 1976). In addition to the light chain heterogeneity studied in the present work, there is evidence of more than one heavy chain species in rabbit skeletal myosin (Weeds, 1967; Starr and Offer, 1973) and in at least one other myosin of nematode origin (Schachat et al., 1977). Pope et al. (1977) have shown that the isolated alkali 1 and 2 types of chymotryptic S1 both contain two species of heavy chain, i.e., light and heavy chain isoenzymes occur independently. The demonstration of light chain homodimers does not therefore imply that a single myosin molecule contains two identical heavy chains.

In studies on the antibody-induced dissociation of DTNB l.c., we observed generally that only about 50% of this light chain could be dissociated (Holt and Lowey, 1975b). This was comparable to the extent of dissociation effected by the thiol reagent DTNB (Hayashi et al., 1973; Kendrick-Jones, 1974), and similar to the situation found in scallop myosin, where EDTA dissociated only 50% of the EDTA l.c. (Kendrick-Jones

et al., 1976). We therefore considered the possibility that, of the two alkali l.c. isoenzymes, one might release all its DTNB l.c. in the presence of homologous antibody, while the other released none. Experiments with papain S1 containing a full complement of DTNB l.c. showed, however, that this was not the case. The unfractionated subfragment and the pure alkali 1 and 2 fractions (Figure 2) were indistinguishable in this respect, with 55–80% of the DTNB l.c. being dissociated by antibody (J. C. Holt and S. Lowey, unpublished results). The affinity of DTNB l.c. for heavy chains is not therefore a property which is linked to the alkali l.c. composition of isolated myosin heads.

The cross-reactivity observed between the difference peptide of alkali 1 and the DTNB l.c. emphasizes the difficulty inherent in using antibodies as specific markers. Cross-reactivity has only recently been demonstrated between alkali 1 and DTNB l.c. (Silberstein and Lowey, 1977), and is consistent with the limited sequence homology between alkali 2 and DTNB l.c. reported by Collins (1976). The difference peptide with its unusual composition and sequence (Frank and Weeds, 1974) was thought to be unique, until experimental evidence proved otherwise. The common determinants are probably located near one end of the DTNB l.c., since tryptic HMM (with a slightly cleaved DTNB l.c.) was successfully fractionated even by difference antibody which cross-reacted with DTNB l.c. Comparison of the N-terminal region of the difference peptide with that of the DTNB l.c. does indeed reveal two common sequences, Pro-Lys-Lys and, four residues distant, Ala-Ala-Ala. These homologies may be the basis of the cross-reactivity as the rest of the difference peptide shows little similarity to DTNB l.c.

Alkali l.c. are generally considered to influence either the ATPase or actin binding properties of myosin. There is, however, no clear understanding of how this might occur, nor of how alkali 1 and 2 might differ in this respect. Specific chemical modification of the alkali l.c. abolished both enzymic and actin binding activities (Wagner and Yount, 1975, 1976) but neither was greatly affected when antibody to alkali 1 was bound to HMM (Holt and Lowey, 1975b; the preliminary results with Fab which were crucial to this conclusion have since been confirmed (J. C. Holt and S. Lowey, unpublished results)). As regards the difference between alkali 1 and 2, studies on the light chain isoenzymes of chymotryptic S1 have revealed a difference only in the actin-activated ATPase measured at low ionic strength (Weeds and Taylor, 1975; Wagner and Weeds, 1977).

An alternative approach is offered by the availability of an antibody marker specific for alkali 1. Applications of this probe include studies on the appearance of alkali l.c. during development (Chi et al., 1975), and the distribution of alkali 1 and 2 in myosin filaments. Preliminary results show that difference antibody binds to myosin in cryostat sections of muscle (G. F. Gauthier, personal communication). Thus one may ask whether alkali 1 myosin is present only in some filaments or, for example, distributed periodically within all filaments. This would be analogous to the work of Craig and Offer (1976) in which the localization of C protein was investigated by labeling thin sections with specific antibody. Studies such as these may be expected to provide new insights on the role and distribution of the alkali l.c. in vertebrate skeletal muscle.

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