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Preparation of the alkali and P light chains of chicken gizzard myosin

Amino acid sequence of the alkali light chain

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1. A simple method is described for the purification of the alkali and P light chains from chicken gizzard myosin. 2. The sequence of the alkali light chain has been unequivocally determined, except for the *N*-terminal dipeptide, by using the tryptic and CNBr peptides. 3. No evidence was obtained for any specific high-affinity Ca^{2+}-binding sites on the alkali light chain. 4. Detailed evidence on which the sequence is based has been deposited as Supplementary Publication SUP 50120 (14 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7QB, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1983) **209**, 5.

Vertebrate muscle myosin is composed of two heavy chains, two phosphorylatable P light chains and two alkali light chains (for review see Grand, 1982). In myosin from smooth muscle the P and the alkali light chains possess molecular weights of 20000 and 17000 respectively and appear to exist as single-gene products. The phosphorylation of the P light chain in chicken gizzard by myosin light-chain kinase is activated at Ca^{2+} concentrations similar to those that initiate hydrolysis of ATP by actomyosin (Sobieszek, 1977*a*). It has been proposed that this phosphorylation step is the triggering mechanism for the contractile process in smooth muscle (Gorecka *et al.*, 1976; Sobieszek, 1977*a*; Chacko *et al.*, 1977), although not all workers agree that there is a strict correlation between the extent of activation of the Mg^{2+}-stimulated ATPase of actomyosin and the P light-chain phosphorylation (Mikawa *et al.*, 1977; Cole *et al.*, 1980, 1982). Less attention has been paid to the study of the 17000-dalton light chain (alkali light chain) and at present there is little information available on the function of this component of the myosin molecule, although Okamoto & Sekine (1980) have shown that it is situated near to the active site of the myosin ATPase.

Kendrick-Jones (1973) originally showed that the P light chain and 17000-dalton light chain of chicken gizzard myosin were similar in amino acid composition to the P and A2 light chains respectively of rabbit skeletal-muscle myosin. Jakes *et al.* (1976) and Maita *et al.* (1981) have extended these observations by demonstrating that more than half of the residues of the amino acid sequences of the smooth- and skeletal-muscle P light chains are identical.

The present work describes a rapid procedure for the isolation of each of the two light chains from smooth muscle and the determination of the amino acid sequence of the alkali light chain. No evidence was obtained for Ca^{2+} binding by the alkali light chain.

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Materials and methods

Preparation of proteins

(*a*) *Myosin.* Myosin was prepared from fresh chicken gizzards essentially by the method of Sobieszek (1977*b*).

(*b*) *Myosin light chains.* Myosin gel from 200g of chicken gizzard, sedimented after 20min centrifugation at 10000*g* in 0.05M-KCl, was dissolved in 5vol. of 7M-urea/10mM-β-mercaptoethanol/50mM-Tris, adjusted to pH 8.0 with 1M-HCl. The following fractionation with ethanol was performed at room temperature (20°C). Ethanol was added slowly to a final concentration of 50%. The suspension was left for 5min and the protein sedimented by centrifugation at 4000*g* for 15min was discarded. Ethanol was added to the supernatant to bring the concentration to 65% and, after being left for 10min, the precipitate was collected by centrifugation at 4000*g* for 15min and used for preparation of the P light chain. Further addition of ethanol to the supernatant, to bring the concentration to 80%, precipitated a fraction that was collected as before and used for preparation of the alkali light chain. Both

light-chain precipitates were redissolved in 50mM-NH_4HCO_3, pH 7.9, dialysed against the same solution in the cold and freeze-dried.

The protein fraction that precipitated between 50 and 65% ethanol contained P light chain contaminated with appreciable amounts of actin and some tropomyosin and myosin heavy chain. These impurities were removed by the following procedure carried out at 4°C. The freeze-dried protein was resuspended in 0.1M-KCl/2mM-$MgCl_2$/1mM-ATP/1mM-dithiothreitol/25mM-Tris, adjusted to pH 8.0 with 1M-HCl (500ml), stirred for 1h and centrifuged at 2000*g* for 10min. Most of the contaminating actin and myosin heavy chains were removed in the precipitate, leaving the light chain in the supernatant, which was dialysed against water and freeze-dried. The freeze-dried protein was redissolved in 5ml of 9M-urea/75mM-Tris/HCl/15mM-β-mercaptoethanol and chromatographed on a column (2.6cm × 120cm) of Sephadex G-100 equilibrated and eluted with 50mM-NH_4HCO_3, pH 7.9. Fractions representing the main peak eluted, which contained the P light chain, were pooled and freeze-dried.

The protein that precipitated between 65 and 80% ethanol was redissolved in 9M-urea/15mM-β-mercaptoethanol/50mM-Tris, adjusted to pH 8.0 with 1M-HCl, and chromatographed on Sephadex G-100, equilibrated and eluted with 50mM-NH_4HCO_3, pH 7.9. The fractions representing the main peak eluted contained the alkali light chain and were pooled and freeze-dried.

Molecular-weight determinations

Molecular weights of the gizzard P and alkali light chains were determined by electrophoresis in the presence of 0.1% sodium dodecyl sulphate at pH 8.3 (0.1-Tris/0.1M-Bicine) on 12% polyacrylamide gels. Transferrin (mol.wt. 77000), bovine serum albumin (65000), ovalbumin (45000), carbonic anhydrase (26000), β-lactoglobulin (18000) and haemoglobin (15000) were used as standards.

Preparation of peptides for sequence determination

(*a*) *Enzymic degradation.* Alkali light chain (30mg) in which the methionine residues had been labelled with iodo[^{14}C]acetic acid (Wilkinson, 1969) was digested with trypsin for 4h at 37°C at an enzyme/substrate ratio of 1:50. The digest was chromatographed on Sephadex G-50 equilibrated and eluted with 50mM-NH_4HCO_3, pH 7.9. The largest tryptic peptide consisting of residues 119–145 eluted as a single component and was freeze-dried. The remaining peptides were further purified by high-voltage electrophoresis. Alkali light chain labelled with iodo[^{14}C]acetic acid at the cysteine residues as described by Wilkinson *et al.* (1972) was also subjected to tryptic digestion and the peptides were purified as described above. Large tryptic and CNBr peptides were digested with thermolysin for 4h or overnight with V8 proteinase at an enzyme/substrate ratio of 1:50 at 37°C. The products of digestion were purified by high-voltage paper electrophoresis.

(*b*) *CNBr digestion.* Alkali light chain (60mg) was dissolved in 70% formic acid (5ml) and a 100-fold molar excess of CNBr over the methionine residues was added. After 24h at room temperature the digest was diluted 10-fold with water and freeze-dried. The CNBr peptides were carboxymethylated at the cysteine residues (Wilkinson *et al.*, 1972) with iodo[^{14}C]acetic acid and chromatographed on a column (2.4cm × 240cm) of Sephadex G-50, equilibrated and eluted with 50mM-NH_4HCO_3, pH 7.9. Peptides were further purified by chromatography on DEAE-cellulose or by high-voltage paper electrophoresis.

Sequencing methods

Manual sequencing methods were essentially as described by Grand *et al.* (1981).

CNBr peptides CB1 (residues 105–146) and CB2 (residues 74–104) were coupled to aminopropyl glass through their *C*-terminal homoserine lactone residues as described by Horn & Laursen (1973). The peptides were sequenced using a Rank Hilger AP524 Automatic Solid Phase Sequencer operated in accordance with the manufacturer's instructions and essentially as described by Laursen *et al.* (1975). Amino acid phenylthiohydantoins were identified by high-performance liquid chromatography on a Spherisorb 3 ODS column eluted with a gradient of 20–60% acetonitrile in sodium acetate, pH 5.0.

Results

Preparation of the light chains of gizzard muscle

Although myosin prepared by the method of Sobieszek (1977*b*) is contaminated with tropomyosin and actin, it is obtained in reasonable yield, enabling, by the procedure described in the present paper, about 20mg of each of the two myosin light chains to be isolated from 100g of chicken gizzard. Modification of the original method for light-chain isolation (Perrie & Perry, 1970) by replacing guanidine hydrochloride with urea to denature the myosin heavy chains increases the possibility of modification (e.g. by cyanation) of the light chains as revealed by multiple bands on electrophoresis in the absence of sodium dodecyl sulphate. This disadvantage, however, can be reduced by minimizing the time of exposure of the light chains to urea and is offset by the ability to separate the two light chains completely by ethanol precipitation. Actin and myosin heavy-chain contamination can be removed from the P light-chain

fraction by centrifugation or by chromatography on DEAE-cellulose in 9M-urea. Although the latter procedure was more effective, it did lead to greater modification of the light chains due to prolonged exposure to urea. For this reason centrifugation followed by gel filtration was normally used for preparation of the light chains and was carried out as rapidly as possible.

The isolated light-chain fractions were at least 95% pure, as judged by electrophoresis at pH 8.3 in 0.1% sodium dodecyl sulphate or at pH 8.6 in 6M-urea. The apparent molecular weights of the P and alkali light chains determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were 18800 and 16500 respectively, compared with 19692 and 16877 calculated from the sequences of the two proteins determined by Maita *et al.* (1981) and reported in the present publication.

Sequence studies on the alkali light chain of gizzard myosin

The sequence of the gizzard alkali light chain is presented in Fig. 1 and the detailed evidence on which it is based has been deposited as Supplementary Publication SUP 50120 at the British Library Lending Division. The deposited data show details of the separation of the tryptic and CNBr peptides by gel filtration. They also show the compositions and electrophoretic mobilities of all the peptides obtained by enzymic and CNBr digestion. Results of the Edman degradations performed on these peptides are also given.

The tryptic digest of the alkali light chain, in which the methionine residues were labelled with iodo[^{14}C]acetic acid, contained 17 peptides of which eight were radioactively labelled. These peptides, which covered the entire sequence of the alkali light chain, were purified by gel filtration of the whole digest on Sephadex G-50, followed by high-voltage paper electrophoresis. The larger fragments were treated with thermolysin or with V8 proteinase and the peptides obtained were purified by high-voltage electrophoresis.

The relative positions of the tryptic peptides were determined by isolation and sequence determination of the peptides obtained by CNBr digestion of the alkali light chain. The largest fragment obtained on digestion with CNBr, peptide CB1, spanning residues 105–146, was found to be a partial cleavage product in which the bond between methionine 119 and threonine 120 had not been split. In sequence studies on calmodulin (Grand *et al.*, 1981), it was observed that a similar sequence was resistant to CNBr digestion. Peptide CB1 was subjected to automated sequence analysis and 13 cycles were completed before it became impossible to identify the amino acid phenylthiohydantoin derivatives with certainty. Peptide CB1 was further digested with thermolysin and V8 proteinase, and the resulting small peptides were isolated, analysed and sequenced manually.

Peptide CB3 (residues 1–35) was found to have a blocked *N*-terminus, as had the parent protein. This fragment was digested with thermolysin and V8 proteinase and the small peptides produced were isolated by electrophoresis. The first 10 amino acids of peptide CB2 (residues 74–104) were determined by automated sequence analysis but identification of subsequent residues was not possible, probably owing to the presence of carboxymethylated cysteine at residue 84. Peptides arising from the digestion of peptide CB2 with V8 proteinase were purified by paper electrophoresis and sequenced manually. Peptides CB4 (residues 36–48), CB5 (60–72), CB6 (49–59) and CB7 (147–150) were also sequenced manually. The presence of methionine in positions 72 and 73 was established by the fact that tryptic peptide T2f2 (residues 63–78) and the thermolysin peptide T2f2ThC1 (residues 69–75) were approximately twice as radioactive and contained twice as much methionine, determined by amino acid analysis, as the other methionine-containing peptides. The four cysteine residues were positioned after purification and partial digestion of the radioactively labelled tryptic peptides obtained after carboxymethylation of the alkali light chain at pH 8.3.

Proton n.m.r. studies

The *N*-terminal-blocking group was established from an examination of the p.m.r. spectrum of the whole alkali light chain and the hexapeptide CB3V8g (residues 1–6). A signal corresponding to the acetyl group was obtained at 2.03 p.p.m. with the whole protein and *N*-terminal hexapeptide derived from CN3 by digestion with V8 proteinase. It was concluded that the *N*-terminus of the alkali light chain is acetylated.

The effect of Ca^{2+} on the three-dimensional structure of the alkali light chain was also studied by p.m.r. At pH 7.2 no perturbation of the p.m.r. spectrum could be observed after titrating from 1mM-$CaCl_2$ (3-fold molar excess of Ca^{2+} over protein) to 3.5mM-EGTA. It was concluded that the light chain did not bind Ca^{2+} under these conditions.

Discussion

The method described for isolation of the light chains of smooth myosin has two major advantages over others described in the literature. The light chains are not chemically modified to facilitate isolation (cf. Maita *et al.*, 1981) and the time spent under the denaturing conditions required for

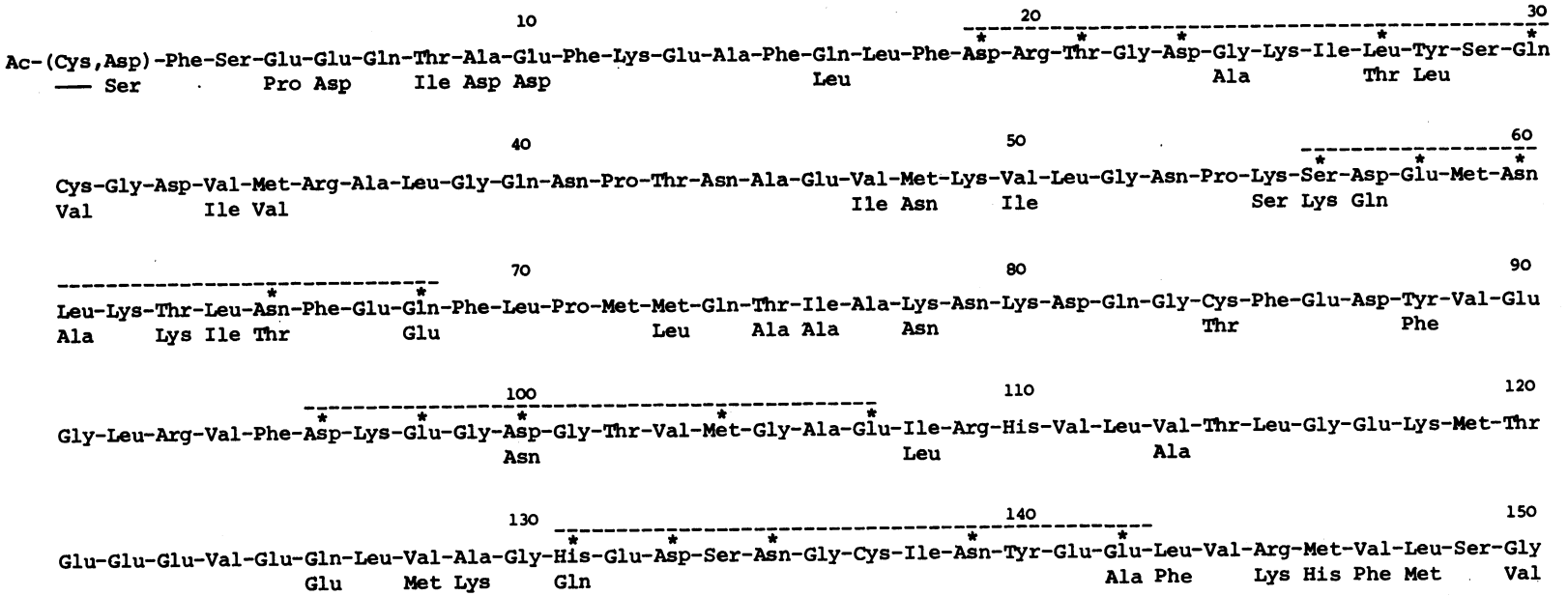

Fig. 1. *Amino acid sequence of the alkali light chain of chicken gizzard myosin*

Putative Ca^{2+}-binding sites are indicated by broken lines above the sequence and those residues that would be expected to be involved in metal ion binding are indicated by asterisks (Kretsinger, 1980). Residues that are different in the sequence of chicken fast-muscle A2 light chains are indicated below the gizzard sequence.

separation from the heavy chain is kept to a minimum, to reduce the extent of spontaneous modification to which these polypeptides are particularly prone.

The alkali light-chain sequence of 150 amino acids has been determined unequivocally, except for the *N*-terminal dipeptide, and is in agreement with the preliminary report published by Matsuda *et al.* (1981), except for the amide assignment at residue 100. In the present work this residue has been determined as aspartic acid. It is quite possible that deamination to aspartic acid could have occurred at some stage during the preparation of either the protein or the tryptic peptides, as has been noted for calmodulin peptides containing Asn-Gly sequences (Grand *et al.*, 1981). The molecular weight of 16877, calculated from the sequence, is in good agreement with that obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the protein has a net charge at pH 7.0 of −15.

The sequence is about 70% identical with the A2 alkali light chains of myosin from chicken and rabbit skeletal muscle (Fig. 1). Also, as is the case with the latter protein, the *N*-terminus of the gizzard alkali light chain is acetylated. The three alkali light chains are of similar length (chicken gizzard alkali light chain, 150 residues; chicken and rabbit fast-muscle A2, 149 residues each), and most of the amino acid substitutions are of a conservative nature. These observations indicate that the sequences of the alkali light chains are more strongly conserved than the P light chains, which in the case of chicken and rabbit skeletal muscle are 53% and 54% identical respectively with that of the corresponding gizzard myosin light chain (Matsuda *et al.*, 1981). The conservation of alkali light-chain structure implies a special significance for the function of this component and underlines the basic similarity of the structure of the myosin molecule in striated and smooth muscle despite differences in the mechanisms for regulation of the two actomysin systems.

The alkali light chain of gizzard myosin is one of a family of proteins of common evolutionary origin that includes the Ca^{2+}-binding proteins calmodulin and troponin C (Barker *et al.*, 1977). The gizzard alkali light chain has 45% of the residues identical with calmodulin and its aromatic amino acid composition is such that its u.v.-absorption spectrum is similar to that of the Ca^{2+}-binding proteins. The major differences in sequence between the gizzard alkali light chain and calmodulin are in the regions of residues 45–80 and residues 125 to the *C*-terminus, where a number of deletions have to be introduced into the sequence.

Application of criteria (Kretsinger, 1980) for the definition of putative Ca^{2+}-binding sites to the alkali light chain sequence of myosin from chicken gizzard indicates that although many of the residues that form ligands for Ca^{2+} in calmodulin and troponin C are conserved, at least one position in each of the four potential Ca^{2+}-binding sites is either occupied by an amino acid residue incapable of acting as a Ca^{2+}-binding ligand, or disrupts the structure in some way (Fig. 1). Although there have been a number of mutations in the Ca^{2+}-binding sites, a large proportion of the hydrophobic residues that Kretsinger (1980) has suggested are necessary for adoption of the 'E-F hand' conformation have been conserved (Grand, 1982). These presumably are of significance for the function of the alkali light chain in gizzard myosin.

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