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Selective cleavage of skeletal myosin subfragment-1 to form a 26 kDa peptide which shows ATP-sensitive actin binding

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A 26 kDa peptide has been cleaved from the C-terminus of the S1 heavy chain with formic acid. Cleavage occurs in the '50 kDa' domain probably at the Asp-600–Pro-601 bond. This fragment has been renatured in the presence of the A2 light chain and the 26 kDa(A2) complex shown to interact with actin in an ATP-sensitive manner.

Myosin; Muscle contraction; ATP binding; Protein fragmentation

1. INTRODUCTION

During the ATPase cycle of muscle, the binding of ATP to myosin serves two major roles. Firstly, it considerably weakens the affinity of the myosin cross-bridges to actin leading to dissociation of the actomyosin complex, implying a change in the conformational (energetic) state of the myosin head [1]. Secondly, after an essentially isoenergetic hydrolysis at the myosin active site, it is in the reattachment to actin, which promotes product release, that the free energy of the reaction is used to perform mechanical work. The nucleotide and actin recognition sites are known to be located on the S1 heavy chain [2] as separate structural entities. Clearly a coupling between these sites must occur to account for the well-known close relationship between the affinities of nucleotides and actin for S1. From a variety of proteolytic and renaturation studies, the myosin head is believed to contain three structural domains: N-27 kDa-50 kDa-20 kDa-C. The location of these sites on these domains of S1 and a knowledge of how they interrelate is necessary for an understanding of the molecular mechanism involved in the energy transduction process.

Here we report on the preparation and actin-binding properties of 26 kDa peptide cleaved from the C-terminus of the S1 heavy chain by formic acid [3], providing a new fragment with which to study the actin-S1-ATP interaction.

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Abbreviations: S1, myosin subfragment 1; S1(A1) and S1(A2), rabbit fast twitch muscle myosin subfragment 1, prepared by chymotryptic digestion and containing either the alkali 1 (A1 or LC1) light chain or the alkali 2 (A2 or LC3) light chain; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of 0.1% SDS

2. EXPERIMENTAL

2.1. *Preparation of muscle proteins*

Fast-twitch muscle myosin was prepared from the longissimus dorsi muscle of New Zealand white rabbits [4] with the modification that KCl (0.6 M, pH 6.8) was used as the extraction medium. S1 was prepared from myosin by chymotryptic digestion [5] as modified in [6]. The isoenzymes, S1(A1) and S1(A2) were isolated on an SP-Tris acryl column [7]. The purified isoenzymes were concentrated against Aquacide II to a concentration of around 10 $mg \cdot ml^{-1}$ and freeze-dried in the presence of sucrose [6] and stored at −20°C. The fast-reacting SH_1 thiol group was labelled with *N*-ethyl-[2,3-^{14}C]maleimide as described in detail elsewhere [6].

Skeletal muscle actin was prepared from rabbit muscle acetone powder [8] and stored at −20°C after freeze-drying as G-actin in 10 mM triethanolamine-HCl, pH 8.0, containing 0.2 mM $CaCl_2$, 0.2 mM ATP and 0.25 mM dithiothreitol. The actin was prepared for use by redissolving in and dialysing

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against this buffer to depolymerise the actin. Any denatured material was removed by centrifugation and the remaining actin was polymerised by dialysing against 10 mM triethanolamine-HCl, pH 8.0, containing 0.25 mM dithiothreitol, 50 mM KCl and 2 mM $MgCl_2$. Alkali light chains were extracted and purified from myosin as described elsewhere [9].

Protein purity and formic acid digestion conditions were monitored by SDS-PAGE in Tris/Bicine buffer, pH 8.3 [10]. Protein concentrations were determined by the microtannin turbidity method [7].

2.2. *Preparation of the 26 kDa (A2) fragment*

A time course of the digestion of S1(A1) (5 mg/ml) in formic acid (70%, v/v) and 6 M guanidine-HCl at 37°C is shown in fig.1A. For preparative purposes the digestion was stopped after 65 h by neutralization with 2 M triethanolamine and the digest was dialysed against 0.2 M triethanolamine-HCl, pH 8, containing 8 M urea (freshly deionised). The heavier fragments precipitated, including some of the 26 kDa fragment, but the supernatant largely contained the 26 kDa fragment and A1 light chain (fig.1B). Further purification was effected by separation on a DEAE-Sepharose column equilibrated with 8 M urea, 20 mM triethanolamine-HCl, pH 8, and eluted by a gradient from 0 to 0.4 M NaCl (over a total volume of 500 ml). The major peak absorbing at 280 nm was collected and found to be the pure 26 kDa fragment; the A1 light chain eluted at a lower ionic strength. A2 light chain was dissolved in the urea/triethanolamine buffer and added to the 26 kDa fragment (2-fold molar excess). After concentration using Aquacide II, the protein complex was renatured by dialysis against 25 mM Mops buffer, pH 7.5, containing 0.2 M sucrose and 10 mM sodium pyrophosphate. The 26 kDa(A2) complex was then separated from excess A2 light chain on a Sephadex G-75 column equilibrated to the renaturation buffer. The eluted complex contained the 26 kDa fragment and the A2 light chain in a 1:1 complex (fig.1C) as judged by scanning the polyacrylamide gel.

2.3. *Binding measurements*

For turbidity measurements in a cuvette, serial additions of S1(A2) (5 μl, 10 μM), 26 kDa(A2) (5 μl, 22 μM) or soybean trypsin inhibitor in the same buffer were made to a solution of actin (1.1 ml, 2 μM) in 10 mM triethanolamine-HCl, pH 8, containing 50 mM KCl. After each addition the contents of the cuvette were mixed and the turbidity of the resulting suspension was immediately noted and recorded for a further 2 min at 400 nm. The rise in turbidity after each S1 or fragment addition occurred instantaneously and no further increase was observed during the 2 min recording. After the final additions of S1(A2) or 26 kDa(A2), ATP (to a concentration of 1 mM) was added. Blanks were obtained by making serial additions of S1(A2), 26 kDa(A2) and soybean trypsin inhibitor to buffer in a reference cuvette. Actin binding was also studied by co-sedimentation of the S1(A2) and 26 kDa(A2) with F-actin in a Beckman airfuge as described in [6,7].

3. RESULTS AND DISCUSSION

3.1. *Preparation of 26 kDa fragment*

A range of preparative conditions were investigated to achieve optimum yield of the 26 kDa fragment. Prior to adopting the conditions outlined above, formic acid digestions were carried out at three different acid concentrations (98%, 80% and 70%) and, in the case of the 70% and 80% concentrations, in the presence or absence of urea (6 M) or guanidine-HCl (4 and 6 M). The acid digestion was also carried out at room temperature and 37°C and over a range of times up to 84 h. When the digestion was carried out with ^{14}C-*N*-ethylmaleimide-SH_1-labelled S1(A1), the radioactivity was retained in the 26 kDa fragment showing that it contained the 20 kDa domain of S1 and that the formic acid cleaved the heavy chain towards the C-terminus of the S1 50 kDa domain, probably at the Asp-600–Pro-601 bond as found earlier by Sutoh [3]. Further evidence for this was provided by showing that the renatured 26 kDa(A2) fragment could be digested with trypsin to yield the radioactive 20 kDa domain and A2 light chain on SDS-PAGE (not shown).

Despite the harsh preparative conditions the 26 kDa fragment appeared to renature readily as shown by (i) its ability to bind the A2 light chain with a 1:1 stoichiometry, (ii) its ability to bind to actin (see below), and (iii) the fact that the 20 kDa domain was still resistant to trypsin treatment. If renaturation was attempted in the absence of the A2 light chain then the resulting 26 kDa fragment was degraded by trypsin into a range of species with molecular masses less than 20 kDa. It should be noted that both the 20 kDa(A2) and 30 kDa(A1 + A2) fragments were also readily renatured in the presence of the light chain after treatment with 6 M guanidine-HCl and ethanol [11,12]. The 26 kDa(A2) complex did not display any ATPase activities, much as expected.

3.2. *Actin-binding properties of the 26 kDa(A2) fragment*

The actin-binding properties of this fragment were demonstrated by either co-sedimentation with actin in the centrifuge or making use of the well recognised increase in turbidity of actin solutions on addition of S1. Both methods not only showed that the 26 kDa(A2) fragment bound to actin but also that this binding was considerably weakened by ATP.

Turbidity measurements are shown in fig.2. Serial additions of either S1(A2) or the 26 kDa(A2)

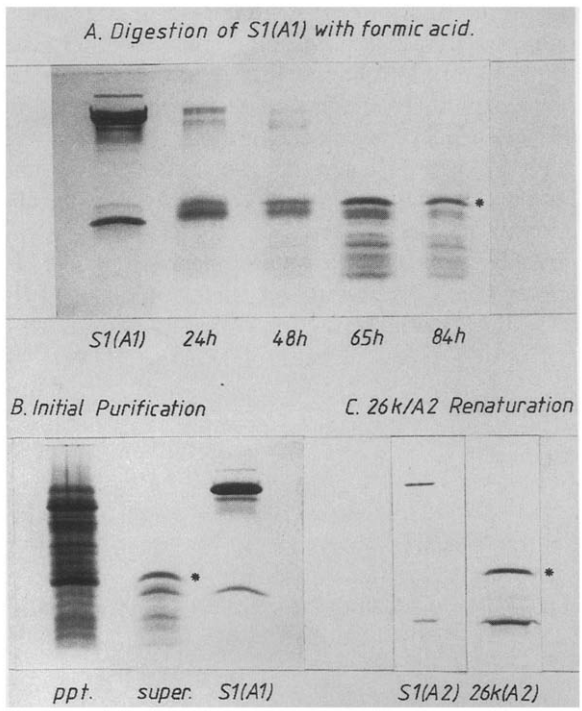

Fig.1. SDS-PAGE of steps in the preparation of the 26 kDa(A2) complex of S1. (A) S1(A1) (5 mg/ml) in formic acid (70%, v/v) and 6 M guanidine-HCl was incubated at 37°C and samples withdrawn at the times indicated for electrophoretic analysis. (B) S1(A1) (20 ml, 5 mg/ml) was digested as in A for 65 h and the digestion stopped by neutralization with 2 M triethanolamine and dialysed overnight against 0.2 M triethanolamine-HCl, pH 8, containing 8 M urea. A precipitate formed, which was removed by centrifugation, and both precipitate ('ppt') and supernatant ('super') analysed by SDS-PAGE. The heavier material dominating the 'ppt' fraction can just be seen in the 48, 65 and 84 h digests in A but is more apparent in this lane as it has been concentrated some 100–1000-fold by centrifugation and sample preparation. (C) The 26 kDa(A2) lane shows this fragment after elution from a Sephadex 9-75 column. The eluted complex contained a 1:1 ratio of 26 kDa fragment and A2 light chain as judged by scanning the polyacrylamide gel. The asterisks denote the 26 kDa fragment.

fragment to actin gave an increase in solution turbidity above that observed when added to buffer alone but this effect was not found with, for example, soybean trypsin inhibitor. On addition of ATP, a decrease of some 50–60% in turbidity was observed for the 26 kDa(A2)-actin and S1(A2)-actin suspensions but no decrease was observed on addition of ATP to the mix of soybean trypsin inhibitor and actin.

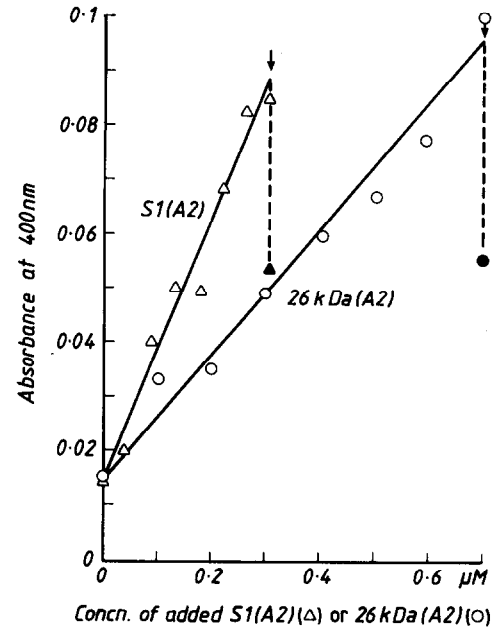

Fig.2. Binding of S1(A2) and 26 kDa(A2) complex to F-actin monitored by an increase in turbidity. Details are given in section 2. The arrows denote the addition of ATP (to a final concentration of 1 mM) and the closed symbols the resulting turbidity measurement.

The data for the co-sedimentation experiments are shown in table 1. The binding of the 26 kDa(A2) fragment to actin was some 100-fold weaker than that of S1(A2) under these conditions. Addition of ATP weakened the affinity of both preparations for actin significantly. Note that the binding experiments in the absence of ATP were carried out at a higher ionic strength, to facilitate the measurements, and so the effect of ATP will be even more enhanced than indicated by the binding constants shown in table 1. When these data were recorded in the form of a Scatchard plot, in all cases the binding stoichiometry was 1 mol of S1(A2) or 26 kDa(A2) binding per mol of actin monomer (actual values varied from 0.87 to 1.15 mol per mol).

The results presented here confirm and extend the observations by Chaussepied et al. [12] who found that a 30 kDa(A2) carboxyl-terminal fragment of S1 prepared by thrombin digestion interacts with actin in an ATP-dependent manner. Like the 30 kDa(A2) preparation, the 26 kDa(A2) complex binds to actin more tightly than the

Table 1

Binding constants for complexes between F-actin and S1(A2) and the 26 kDa(A2) preparation

F-actin complexes with	Binding constant (M^{-1})	
	− ATP	+ ATP (3 mM)
S1(A2)	9.6×10^6	2.3×10^4
26 kDa(A2)	1.1×10^5	2.5×10^3

Binding constants were determined by co-sedimentation of the acto-protein complex as described in [7]. The concentration of S1(A2) and the 26 kDa(A2) fragment derived from it were determined by radioactive counting of the *N*-ethyl-[^{14}C]maleimide-SH_1-labelled material. The experiments were carried out in 10 mM triethanolamine-HCl, pH 8.0, containing 3 mM $MgCl_2$ and 0.25 mM dithiothreitol and either 100 mM KCl (− ATP) or 10 mM KCl (+ 3 mM ATP)

20 kDa(A2) domain [11,12], possibly suggesting that an actin-binding site exists on the 6 kDa C-terminal region of the 50 kDa domain in addition to that on the 20 kDa domain [11,13]. The most important observation, however, is that the 26 kDa(A2) and 30 kDa(A2) [12] fragments bind to actin in an ATP-sensitive manner, unlike the 20 kDa(A2) fragment. Thus, these longer portions of the S1 molecule exhibit a crucial feature of native myosin heads. Chaussepied et al. [12] also showed that actin dissociation is consequent only on the attachment of the polyphosphate chain of ATP to the 30 kDa fragment, and by implication to the C-terminal 10 kDa region of the 50 kDa domain. Our results now locate this region that contains the β-phosphoryl subsites (and possibly additional actin-binding subsites) to the C-terminal 6 kDa fragment of the 50 kDa domain, providing an even smaller allosteric unit of the myosin head for further study.

Photoaffinity labelling of S1 with entrapped ATP analogues and sequence comparisons with other ATP-binding proteins have shown that both the 27 kDa domain [14,15] and the N-terminal region of the 50 kDa domain [16] are involved in binding the adenine and ribose portions of ATP. Image reconstruction of electron micrographs [17] also locates the adenine subsite of ATP on the opposite side of the head to the SH_1 thiol group and actin-binding site. In the presence of ATP, however, the SH_1 thiol can be cross-linked to a Cys in the C-terminal of the 50 kDa domain [18], implying that these regions are on the same side of the myosin head. The balance of data, then, points to ATP binding in a cleft between the 27 kDa and 50 kDa domains, 'phosphate first', placing the adenine nearier to one side of the head and the phosphate closer to the other; nearer, in fact, to the proposed actin-binding site(s) on the 50 kDa and 20 kDa domains.

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REFERENCES

[1] Bagshaw, C.R. and Trentham, D.R. (1973) Biochem. J. 133, 323–328.
[2] Wagner, P.D. and Gininger, E. (1981) Nature 292, 560–561.
[3] Sutoh, K. (1983) Biochemistry 22, 1579–1585.
[4] Trayer, I.P. and Perry, S.V. (1966) Biochem. Z. 345, 87–100.
[5] Weeds, A.G. and Taylor, R.A. (1975) Nature 257, 54–56.
[6] Trayer, H.R. and Trayer, I.P. (1983) Eur. J. Biochem. 135, 47–57.
[7] Trayer, H.R. and Trayer, I.P. (1988) Biochemistry 27, 5718–5727.
[8] Spudich, J.A. and Watt, S. (1971) J. Biol. Chem. 246, 4866–4871.
[9] Henry, G.D., Winstanley, M.A., Levine, B.A. and Trayer, I.P. (1985) Biochim. Biophys. Acta 830, 233–243.
[10] Weeds, A.G., Hall, R. and Spurway, N.C. (1975) FEBS Lett. 49, 320–324.
[11] Chaussepied, P., Mornet, D., Audemard, E., Kassab, R., Goodearl, A.J., Levine, B.A. and Trayer, I.P. (1986) Biochemistry 25, 4540–4547.
[12] Chaussepied, P., Mornet, D. and Kassab, R. (1986) Biochemistry 25, 6426–6432.
[13] Griffiths, A.J., Levine, B.A. and Trayer, I.P. (1987) Biochem. Soc. Trans. 15, 874–875.
[14] Mahmood, R., Cremo, C., Nakamaye, K.L. and Yount, R.G. (1987) J. Biol. Chem. 262, 14479–14486.
[15] Walker, J.E., Saraste, M., Runswicke, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.
[16] Okamoto, Y. and Young, R.G. (1985) Proc. Natl. Acad. Sci. USA 82, 1575–1579.
[17] Tokunaga, M., Sutoh, K., Toyoshima, C. and Wakabayashi, T. (1987) Nature 329, 635–638.
[18] Ue, K. (1987) Biochemistry 26, 1889–1894.