CHAIN REGISTER IN MYOSIN ROD

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1. Introduction

Myosin is an important component of the contractile apparatus of both muscle and non-muscle cells. The molecule consists of two globular heads, which interact with actin, and a long rod-like tail which, in muscle cells, forms the backbone of thick filaments. The tail portion of the myosin molecule, generally referred to as myosin rod, can be prepared by proteolysis [1] and is constructed from two identical or near-identical [2], α-helical chains arranged in a coiled-coil conformation [3,4]. By analogy with studies on the other fibrous contractile proteins, paramyosin [5] and tropomyosin [6–8], the chains are thought to be in register. However, firm experimental evidence for this proposal has not been obtained. A great deal of myosin sequence information is now becoming available and in order for it to be analysed, it is vital that the relative position of the chains should be known.

In this article it is demonstrated that disulphide bonds can be formed between the chains within a portion of the myosin rod molecule, which establishes that these chains are in register.

2. Materials and methods

Myosin was prepared from rabbit back and thigh muscles and rod obtained by digestion with chymotrypsin in 0.12 M NaCl as in [9]. Disulphide bonds were produced, similarly to tropomyosin [6], by oxidising rod at a ~4 mg/ml in 0.6 M NaCl, 10 mM Tris–HCl (pH 8) with 5 mM 5,5′-dithio-bis-(2-nitro-benzoic acid) (DTNB), then dialysing exhaustively to remove unreacted DTNB.

Short S2 was prepared from oxidised rod by digestion with trypsin in 0.6 M NaCl, 10 mM Na-phosphate buffer (pH 7) for 30 min at 25°C at enzyme/substrate = 1:50. After precipitation of light meromyosin by dialysis to 30 mM NaCl, 5 mM Na-phosphate (pH 6.5) the short S2 was separated from other fragments by gel filtration in 0.1 M NaCl, 25 mM Na-borate buffer (pH 8) at 4°C using a 95 × 2.6 cm column of Sephacryl S-300 operated at 10 ml/h and taking 5 ml fractions.

Cross-linking was done at ~1 mg protein/ml in 0.1 M NaCl, 50 mM Na-borate (pH 8) at room temperature with 4 mM glutaraldehyde. After 30 min the reaction was quenched by the addition to 0.1 M Tris–HCl (pH 8) which rapidly reacted with any remaining aldehyde groups.

Ultracentrifugation was carried out in 0.1 M NaCl, 25 mM Na-borate (pH 8) using a Beckman model E ultracentrifuge equipped with Rayleigh interference optics and operated at 16 000 rev./min at 20°C. The partial specific volume of short S2 was taken as 0.728 [1].

SDS electrophoresis was carried out using 8% polyacrylamide mini gels and a 0.1 M Tris–bicine (pH 8.1), 0.1% SDS running buffer. Gels were stained with Coomassie brilliant blue and destained in methanol–acetic acid–water mixtures.

3. Results and discussion

Treatment of reduced rod with DTNB resulted in the production of thionitrobenzoate ions corresponding to 5 cysteine residues/chain of rod, which is in excellent agreement with [2]. As was observed with tropomyosin [6], disulphide bonds rapidly formed between modified cysteine residues in this material and, after dialysis for 24 h to remove unreacted DTNB, <0.1 mol thionitrobenzoate/chain of rod was released when made 2 mM with dithiothreitol. Thus, the formation of disulphide bonds between chains was essentially quantitative.

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The high initial M_r of rod and its marked tendency to self-associate made direct investigation of the disulphide bonds difficult. For this reason, further work concentrated on the well-characterised tryptic fragment of the myosin rod generally referred to as 'short S2'. Each chain in this fragment contains 2 cysteine residues and corresponds to ~320 residues of the rod sequence adjacent to the head [10]. This fragment retains the α-helical, coiled-coil configuration of the rod [11], but, unlike other rod fragments, it does not usually self-associate [12]. To ensure that the chains in this fragment were in the same register as in the parent rod, the material employed in this study was produced from rod in which the disulphide bonds had already been formed.

Polyacrylamide–SDS gel electrophoresis of oxidised short S2 (fig.1b) indicated ~68 000 M_r, whereas samples that had been incubated subsequently with 15 mM 2-mercaptoethanol (to reduce any disulphide bonds present) showed ~34 000 M_r under these conditions (fig.1c). This halving of M_r in the presence of mercaptoethanol established clearly that disulphide bonds were present in the oxidised material.

The formation of disulphide bonds either within a single molecule or between chains from different molecules would produce an increased M_r in the SDS gel electrophoresis system and so it was important to establish which type of bond was present. This was done by examining oxidised short S2 in non-denaturing systems, where the M_r would be expected to correspond to 2 chains (68 000 M_r) if the bonds were all within a molecule, but to ⩾4 chains (>136 000 M_r) if there were bonds between molecules.

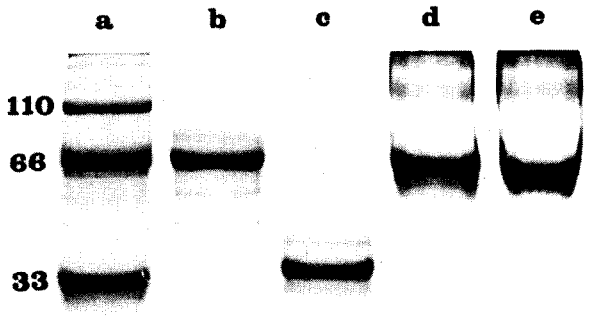

Fig.1. Electrophoresis patterns for short S2: (a) M_r standards (myosin rod 110 000 M_r, oxidised rabbit skeletal tropomyosin 66 000 M_r, reduced rabbit tropomyosin 33 000 M_r); (b) oxidised short S2 68 000 M_r; (c) reduced short S2 34 000 M_r; (d) oxidised, cross-linked short S2; (e) reduced, cross-linked short S2. Most of the material in the cross-linked material was 68 000 M_r but in both instances a small quantitity of material of ~140 000 M_r (4 chains) was also present.

Relative molecular masses in a non-denaturing system were determined by the high-speed sedimentation equilibrium method [13]. That obtained for oxidised short S2 was 69 400 ± 5000 while that for reduced short S2 (examined under the same conditions as the oxidised material except that 2 mM dithiothreitol was also present) was 67 500 ± 5000. Both values were those expected for 2-chain aggregates and were in good agreement with previous values for reduced material [1]. Further evidence that all the disulphide bonds in the oxidised short S2 were intramolecular was obtained by cross-linking with glutaraldehyde. Under the conditions employed, glutaraldehyde formed cross-links mainly between the chains within a single molecule, as shown by most of the reduced cross-linked short S2 having 68 000 M_r when examined by polyacrylamide–SDS gel electrophoresis (fig.1e). A small number of cross-links were also formed between molecules as evidenced by the presence of a small amount of ~140 000-M_r material also being present in the reduced sample. The pattern derived from oxidised cross-linked short S2 also contained mainly 68 000-M_r material (fig.1d). This would happen only if the disulphide bonds were entirely between chains within molecules because disulphide bonds between molecules would have produced cross-linked material with ⩾136 000 M_r.

The formation of disulphide bonds between the chains within a single molecule established that the chains in the short S2 fragment must be in register, since steric hindrance makes it impossible to form these bonds when the chains are staggered [14]. And since the entire rod portion of myosin is probably a continuous α-helical, coiled-coil [11], this implies very strongly that the chains are in register along the entire length of the rod portion of the molecule. Of course it could be argued that, since there are two cysteine residues in short S2 [10], the chains might be staggered so that the disulphide bonds were not formed between the same residue in each chain. However, this is very unlikely because:

(i) Only half of the cysteine residues should be able to form disulphide bonds under these circumstances whereas the formation of disulphides with DTNB was essentially quantitative;

(ii) The sequence data available on rabbit short S2 [15] shows that these two residues must be separated by $\geqslant$140 residues, which is much greater than any likely value of chain stagger.

In addition to providing a more reliable basis for analysis of myosin sequence data, the present results also raise two interesting points with respect to the general structure of myosin:

(i) Although in the analysis [16] of fragments of the rabbit myosin sequence, a similar pattern of salt bridges were claimed in positions *e* and *g* of the coiled-coil as were found in tropomyosin [14], close analysis of the rabbit data [15,17] shows that these salt bridges are extremely infrequent and are certainly much less abundant than in tropomyosin. Furthermore, analysis of the sequence of nematode *unc*-54 myosin (kindly communicated by J. Karn and A. D. McLachlan) shows a similar low frequency of these salt bridges. Therefore, it would seem unlikely that the register of the myosin chains is maintained in this way. The size of the residues at the interface between the two chains may possibly have a role here.

(ii) In a molecule in which the chains in the rod are in register, the two myosin heads will be related by a 2-fold rotation axis. The two heads of the molecule cannot preserve this symmetry and still interact in an equivalent manner with a single thin filament. But the degree of strain which would have to be introduced into the molecule to overcome this non-equivalence need not be large and could, for example, involve no more than the rotation of one head by 180° accompanied by a small degree of translation. This might easily be taken up by, say, one of the flexible regions of the molecule, but it does, none the less, impose a further constraint on the conformation of a cross-bridge in vivo.

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