THE MYOSIN DIMER: AN INTERMEDIATE IN THE SELF-ASSEMBLY OF THE THICK FILAMENT OF VERTEBRATE SKELETAL MUSCLE

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1. Introduction

The participation of a parallel myosin-dimer as a potential intermediate in the self-assembly of the thick-filament of a vertebrate skeletal muscle has had a long, contentious history. The first indication of its presence came from ultracentrifuge studies on myosin solutions at high ionic-strength [1] where a 'monomer–dimer' equilibrium was reported. Under these conditions myosin had been considered a monomer. Laser light scattering experiments [2] provided independent though not conclusive support for [1]. The dimer subunits were considered to lie parallel to each other [3] and to have an axial stagger of 43 nm this being the measurement of the true axial repeat of the subunits of the native thick filament [4]. Considering the available evidence, it was an arbitrary assignment of a dimension, as the axial stagger of the dimer could equally well have related to a multiple of the 14.3 nm axial translation [4] of the filament subunits and not to the true axial repeat of 43 nm (3 × 14.3 nm). Later hydrodynamic [3] and chemical cross-linking experiments [5] failed to answer the question unequivocally. The existence of the dimer was questioned [6] in a reinvestigation of the monomer–dimer equilibrium in the ultracentrifuge. They attributed the variation seen in s_0 to a conformational change in head region of the myosin molecule and not to a monomer–dimer equilibrium. The above experiments were all done under conditions where myosin filament formation is inhibited by the high concentration of salt.

The first direct evidence for the participation of a dimer in the self-assembly of the thick-filament came from experiments in which the reassembly of pressure-dissociated myosin into filament was found to be analysable in terms of a bimolecular reaction between 'like' species [7]. The assembly reaction was later shown to occur via a two-step mechanism in which the two 'like' species were found to be in rapid pre-equilibrium with a dimer, which in turn adds on to the growing filament in a rate limiting reaction [8].

$$A + A \overset{\text{rapid}}{\longleftrightarrow} B + \text{Fi-2} \overset{\text{slow}}{\longrightarrow} \text{Fi}$$

The experiments described here were devised to give physical identity to the dimer under conditions where it is known to participate in filamentogenesis [7–9]. Hydrostatic pressure was used to dissociate the myosin filaments. It was hoped that the increase in the concentration of non-filamentous myosin would lead to an increase in dimer concentration above that seen at atmospheric pressure. Chemical cross-linking [5] was used to 'freeze' the structures present at pressure for later examination by electron microscopy. It was hoped that a combination of the change in concentration of non-filamentous myosin and the increase in hydrostatic pressure would alter the distribution of the intermediates present in a specific and identifiable way.

2. Experimental

2.1. *Filament preparation*

Myosin was prepared from the back and hind-leg muscles of a rabbit [9]. The filaments were generated from purified myosin by a 2 × 16 h dialysis against a KCl–bicine buffer solution. The ionization of bicine and hence the pH of the buffer is virtually insensitive to changes in hydrostatic pressure [9].

2.2. *Pressure-jump apparatus*

The instrument used is described in [1,9].

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2.3. *Electron microscopy*

The myosin was prepared for replica making by the method in [12] as modified in [13]. Platinum was rotary shadowed onto the sample at an angle of 6°. The material was examined in a JEM 100S electron microscope at a magnification of 34 000. A digital measuring device (Digiplan, Kontron Messergate GMBH Munich) was used to measure the dimensions of the photographic prints of the dimer micrographs. The lengths measured were corrected for the thickness of the platinum shadow by subtracting 1 nm [12,13].

2.4. *Materials*

The chemicals used were of Analar grade and were generally obtained from BDH Chemicals (Poole, Dorset). The glutaraldehyde used was a 25% solution prepared specially for electron microscopy by BDH. Bicine was obtained from the Sigma Chemical Co. (St Louis MO).

3. Results and discussion

3.1. *Cross-linking and dimer*

The myosin filament–monomer equilibrium was generated by dialysis against 0.150 M KCl, 0.005 M bicine buffer, pH 8.1 at 5°C [9]. A graph of filament turbidity against pressure for this material is shown in fig.1. The linear decrease in turbidity is directly proportional to weight lost from the filaments as they progressively shorten towards the bare-zone at the centre of the thick-filament [9]. Filament dissociation is virtually complete at 20 MPa, the point of transition from the pressure-sensitive to the pressure-insensitive phase (see fig.1).

Here, the structures present at various degrees of filament dissociation were 'frozen' by chemical cross-linking with glutaraldehyde for later examination in the electron microscope [5]. The kinetics of the cross-linking reaction were first established at atmospheric pressure by following the extent of filament cross-linking as in [5]. A 30 min reaction time, with glutaraldehyde present in a 300-fold molar excess over myosin monomer, sufficed to cross-link the filaments to the extent that they no longer dissociated in solutions of high ionic-strength. The experimental procedure used was as follows: A glass syringe was filled with 3 ml solution containing 1.25 mg myosin/ml, 0.150 M KCl, 0.005 M bicine (pH 8.09) at 5°C and a 1 ml air bubble. Glutaraldehyde, suitably diluted with 0.150 M KCl to a final volume of 75 μl, was rapidly

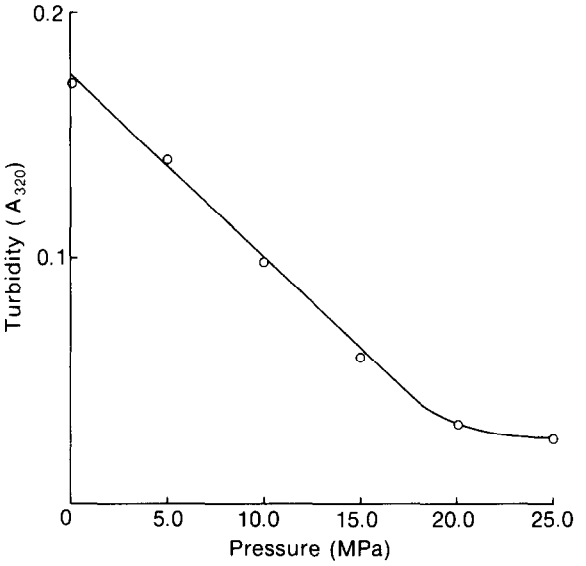

Fig.1. The effect of hydrostatic pressure on the turbidity of a myosin filament solution. The filaments are virtually fully dissociated at a pressure of 20 MPa. Myosin was 1.25 mg/ml.

injected into the syringe containing the myosin. The cross-linking reaction was initiated by inverting the syringe a few times. The syringe contents were then rapidly injected into the pressure-jump cell, the taps closed and pressure applied. At the end of 30 min the cell was depressurised and the cross-linking and reassembly reactions halted by rapidly mixing the sample with an equal volume of 1.2 M ammonium acetate (to present filament reassociation) and 2.5 mM NaCNS (to inactivate the glutaraldehyde). The cross-linked myosin was then prepared for electron microscopy (see section 2).

3.2. *Dimer distribution under pressure*

The amount of dimer present at a particular pressure was determined by counting all the structures that were recognisable as either dimer or monomer. The relative distribution of the two species was recorded as the percentage of monomer present as dimer over the total amount of monomer present both as dimer and monomer.

The pressure dependence of the relative abundance of dimer is illustrated in fig.2. The distribution curve is biphasic and exhibits some interesting features. At first the dimer concentration rises towards a peak at 7.5 MPa. Dimer generated from myosin monomer by either chance of specific bimolecular interaction would exhibit this type of behaviour in response to a

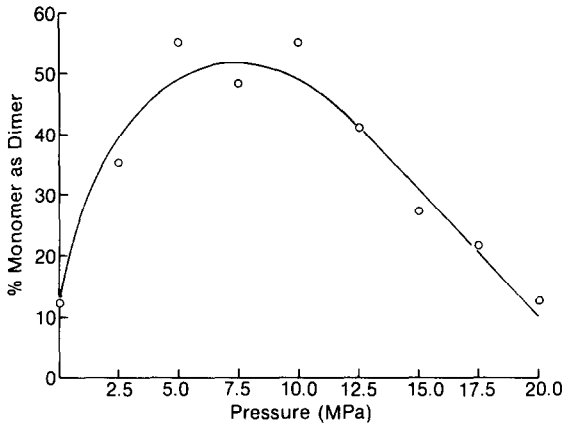

Fig.2. The distribution of cross-linked dimer with pressure: 100 structures were counted at each pressure.

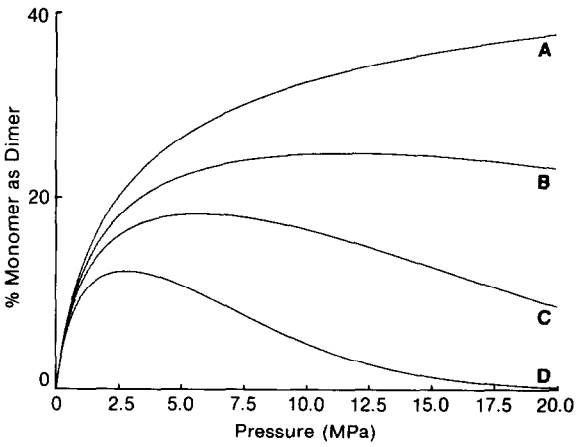

Fig.3. Computer simulation of a monomer–dimer equilibrium showing various degrees of pressure sensitivity. The equilibrium constant was 2×10^6 M^{-1} at atmospheric pressure. Myosin was introduced linearly with pressure to a final concentration of 2×10^{-6} M at 20 MPa. The ΔV° in ml/mol dimer dissociated for the various reactions was: (A) 0; (B) −200; (C) −400; (D) −800.

linear increase in the concentration of non-filamentous myosin. However, at >7.5 MPa the relative dimer concentration declined steadily to a low value at 20 MPa, the pressure at which the filaments are virtually fully dissociated [9]. A simple monomer–dimer equilibrium would not behave in this way. It was thought likely that this decrease in dimer concentration was due to the increase in the non-filamentous myosin concentration being insufficient to overcome the pressure-induced increase in the dissociation constant. The kinetics of the cross-linking reaction between the aldehyde groups of glutaraldehyde with the ϵ-amino groups of lysine should show little pressure sensitivity; the mechanism being essentially a substitution reaction [14].

3.3. *Specificity of the dimer cross-linking reaction*

Computer simulation of a similar system revealed certain interesting features (see fig.3). The pressure dependence of the dimer distribution (given as the percentage of monomer as dimer over the total myosin concentration) was calculated for an assembly reaction with ΔV° values ranging from 0 to −800 ml/mol dimer dissociated. The myosin concentration increased linearly with pressure (see fig.1). The simulated reactions of fig.3 having a marked pressure sensitivity bear the closest resemblance to the dimer distribution seen in fig.2. Thus the influence of pressure in the dimer distribution should be primarily due to the direct effect of pressure on the assembly reaction and not to the indirect effect of pressure on the rate of the cross-linking reaction. The monomer–dimer equilibrium studied at high salt in the ultracentrifuge showed no detectable pressure sensitivity [1].

The marked pressure-sensitivity of the dimer assembly reaction does in fact provide good evidence to support the thesis that most of the dimers seen are the product of the specific interaction of myosin molecules. This follows from the observation that it is only possible to obtain a large ΔV° of solvation by the cooperative clustering of a number of pressure-sensitive groups to form an extensive recognition/contact zone at the interface between the dimer subunits. The major contribution to the pressure-sensitivity of reactions between protein chains comes from the solvation of ionic amino acid residues (the average ΔV° of solvent being −15/mol each [1]). Chance cross-linking by contrast would at most involve the contact of a few amino acid side-chains; the nett consequence being that such reactions would show little pressure sensitivity.

3.4. *The dimer structure*

The dimer micrographs are of materials from the peak of the pressure-dependent distribution curve (7.5 MPa, see fig.2). The structures seen can be classified into three classes, depending on whether they clearly show; (i) parallel (head-to-head); or (ii) anti-parallel (tail-to-tail) contact for a part of their length; or (iii) whether they appear to be attached by the tip of the tail of one subunit to the rod section of

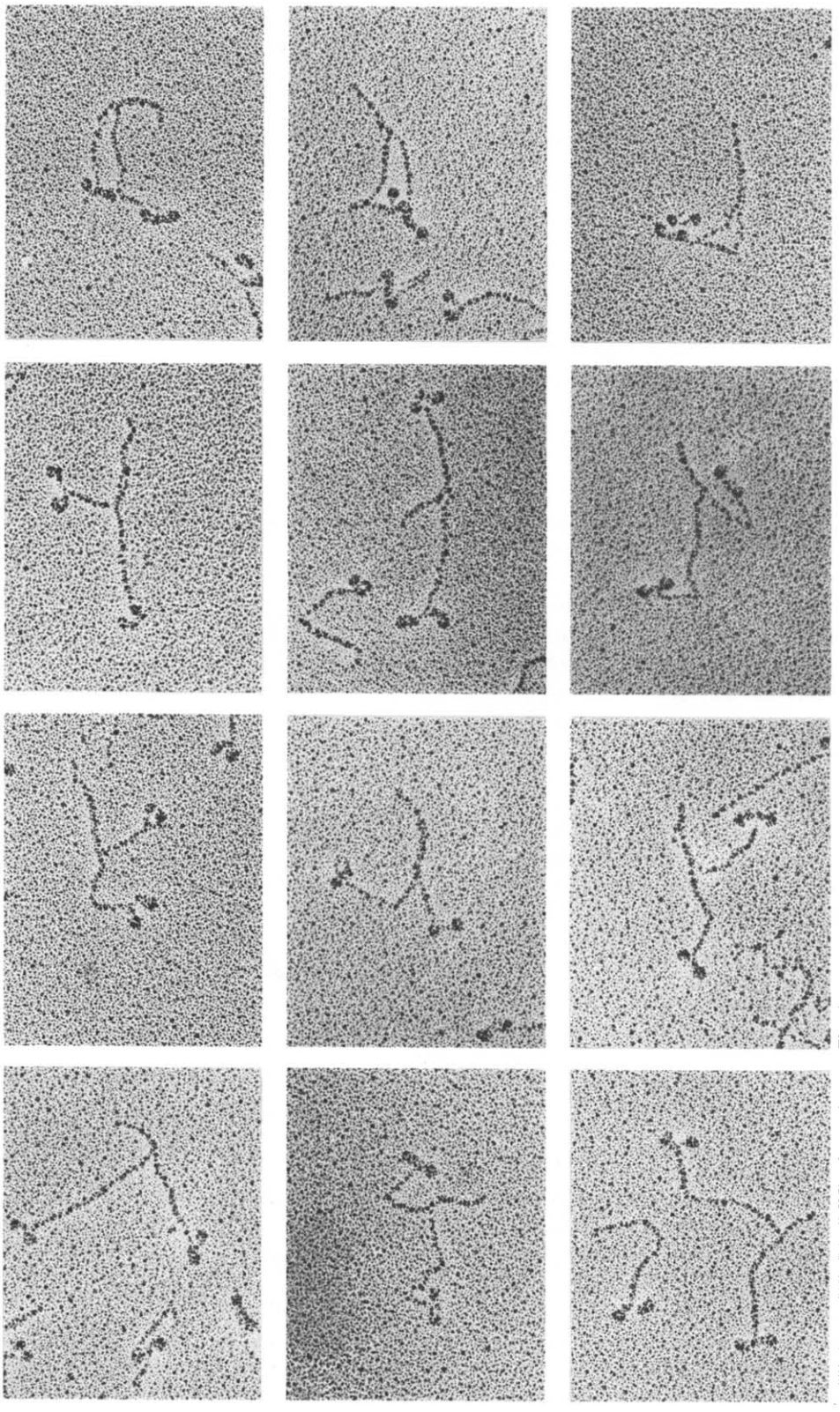

Fig.4. Electron micrographs of the parallel dimer of myosin having a mean axial stagger of 44 nm. The bar represents 200 nm.

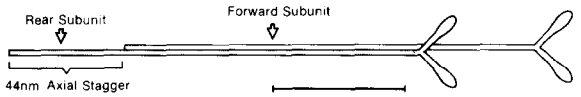

Fig.5. A scale drawing of the parallel dimer intermediate in filamentogenesis. The bar presents 50 nm.

the other. With class (iii) it is difficult to distinguish whether cross-linking is a result of the parallel or anti-parallel interaction of subunits.

The predominant species seen (73%) proved to be a parallel dimer with a mean axial stagger of 44.2 nm SD = 7.7 nm (n = 73). Contact between the individual subunits occurred by either parallel or tip-of-tail interaction (see fig.4). The rear subunits had a tail length of 154 nm, SD = 5.7 nm (n = 73); the tail length of the forward subunit being 151 nm SD = 6.2 nm (n = 7.3). Myosin monomer shadowed together with the dimer was found to have a tail length of 155 nm SD = 6.2 nm (n = 108). This dimension is in close agreement with that reported by other workers, namely 156 nm SD = 5 nm [15] and 157 nm SD = 7 nm [13]. Whether the shorter tail lengths of the dimer subunits are an artefact or are the result of a structural change on dimerization is unclear. A model of the dimer, using the dimensions of the myosin molecule obtained in [16], is shown in fig.5.

The second distinctive species (8%) was a tip-of-tail to tip-of-tail (presumably anti-parallel) dimer. The remainder (17%) consisted of dimers having an axial stagger of 70–110 nm and exhibiting all 3 classes of attachment between the subunits. These dimers formed a dimensionally separate group from that of the 44 nm parallel dimer.

4. Conclusion

A method has been devised to distinguish between chance and specific interactions in the cross-linking of macromolecular systems with pressure-sensitive equilibria. The technique was used to provide evidence to support the contention that the parallel myosin dimer with an axial stagger of 44 nm is in fact the filamentogenic dimer seen in the kinetic experiments on myosin filament assembly [7,8]. It is difficult to tell whether it is the same as the dimer thought to be present at high salt concentrations [1,3]. However, the two certainly seem to differ in their sensitivity to hydrostatic pressure; this could be due to the filamentogenic dimer having a more extensive area of contact between the subunits (section 3).

The axial stagger observed, seems without doubt to relate to the true 43 nm cross-bridge repeat of the myosin thick filament of vertebrate skeletal muscle. Thus the function of the 44 nm dimer as the basic building block of the thick filament of vertebrate skeletal muscle after the central bare-zone has been formed seems well established. The report [7] of an axial stagger of 76 nm SD = 16.2 nm for the kinetic dimer was made on the basis of low resolution micrographs.

Acknowledgements

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