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Pressure-jump studies on the length-regulation kinetics of the self-assembly of myosin from vertebrate skeletal muscle into thick filament

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The self-assembly of myosin monomer into thick filament occurs via a two-step mechanism. At first a pair of myosin monomers reacts to form a parallel dimer; the dimer in turn adds to the filament ends at a rate that is independent of filament length. The rate of the dissociation reaction on the other hand is length-dependent. The 'off' rate constant has been shown to increase exponentially by a factor of 500 as the filament grows from the bare-zone out to its full length. The length of the filament is thus kinetically controlled; myosin is added to the filament at a fixed rate, whereas the dissociation rate increases to a point where equilibrium is established and the filament ceases to grow. The structural implications implicit in the mechanism are discussed.

The pressure-jump experiments on the equilibrium-thermodynamic behaviour of thick filaments generated *in vitro* posed a number of questions best answered by a study of the dynamics of the individual association and dissociation reactions (Davis, 1981). In the preceding paper (Davis, 1981), it was established that an increase in hydrostatic pressure caused the myosin thick filaments to shorten symmetrically about the bare-zone presumably by the loss of subunit from the filament ends. The mechanism of length regulation that gave rise to the relatively monodisperse population of filaments at atmospheric pressure (Josephs & Harrington, 1968) continued to operate as the filaments shortened towards the bare-zone. It was, however, not possible to elucidate the underlying mechanism responsible for these phenomena.

The first indication that the kinetics of the self-assembly reaction could be studied in the pressure-jump emerged from a preliminary investigation in which it was shown that two like species reacted in a second-order step at some stage on the filament self-assembly pathway to form a dimer (Davis & Gutfreund, 1976). The recent discovery of a parallel dimer of myosin under identical experimental conditions with those used in the present paper has given a structural identity to the 'kinetic' dimer (Davis *et al.*, 1981). The dimer has a stagger of 76 nm twice the previously predicted value of 43 nm reported by Godfrey & Harrington (1970*a*,*b*) and Reisler *et al.* (1973).

A kinetic analysis of the self-assembly reaction offers an opportunity to pinpoint the stage in the mechanism at which the co-operativity responsible for the regulation of filament length operates. Mechanisms for both the assembly and disassembly of the thick filament *in vitro* are proposed and discussed in detail.

Materials and methods

General

Rabbit back and hind-leg muscle was used as a source of myosin. The purification procedure, the preparation of the filament equilibria, the myosin concentration measurements and the chemicals used were as described in the preceding paper (Davis, 1981).

Concentration measurements

In kinetic experiments the contribution to the observed turbidity signal by non-filamentous myosin, albeit small (some 6% of the total), should be taken into account. The specific molar turbidities obtained for filament and salt-dissociated myosin (Davis, 1981) were used to calculate a specific molar turbidity for the transfer of monomer subunit (M) into filament (F) and vice versa. The value was obtained as follows:

$$\underset{\varepsilon_{320}}{\mathrm{M}\rightarrow\mathrm{F}} = \underset{\varepsilon_{320}}{\mathrm{F}-\mathrm{M}} = (6.93\times10^{4})-(4.19\times10^{3})$$
$$= 6.51\times10^{4}\ \mathrm{litre\cdot mol^{-1}\cdot cm^{-1}}.$$

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The pressure-jump apparatus

The basic design of the pressure-jump cell and its accessory electronic and optical components have been described elsewhere (Davis & Gutfreund, 1976; Davis, 1981).

Data manipulation

In kinetic experiments the relaxations were converted from analogue into digital form and stored in the memory of a transient recorder (Datalab DL905; Data Laboratories, Mitcham, Surrey, U.K.) as 1 kilobyte of 8 bit binary data. These data were transferred to the memory of a 48K Appleplus II micro-computer (Apple Computer Inc., Cupertino, CA, U.S.A.) via an EPROM (Erasable Programmable Read Only Memory)-controlled PIA (Peripheral Interface Adapter). The transient and the end-point of the reaction were stored as a 2 kilobyte binary file on a floppy diskette (Disk II; Apple Computer Inc.). The transmission signal was converted into an absorbance signal for display on the video monitor. The absorbancy record was converted into the concentration of monomer added to or lost from the filament using the specific turbidity value for the transfer of monomer to filament. The type of kinetics found dictated the linearization procedure and the rate constant was obtained from the slope of the derivative by the use of a least-squares fitting procedure. A hard copy of the various graphs could be obtained by the use of a digital plotter (Miplot WX 471; Watanabe Instruments Corp., Tokyo, Japan).

Results and discussion

Filament assembly

The kinetics of the assembly of the thick filament from myosin monomer can be analysed by standard kinetic techniques (Davis & Gutfreund, 1976). The filaments present at atmospheric pressure were partially dissociated by the application of hydrostatic pressure. After the new equilibrium had been established the assembly reaction was initiated by a stepwise return to atmospheric pressure in some 60μs. A direct measure of the amount of monomer subunit incorporated into filament was obtained by recording the change in the turbidity of the solution at 320nm with time (see the Materials and methods section). The kinetics were analysed in terms of a rate-limiting second-order reaction in which two like species of unknown structure react together at some stage on the assembly pathway (Davis & Gutfreund, 1976). The relaxation amplitude has to be set between two limits for the kinetics to be analysable. The extent of filament dissociation has to be sufficiently large so that the reverse reaction has a negligible effect on the overall kinetics; however, at the same time the extent of the

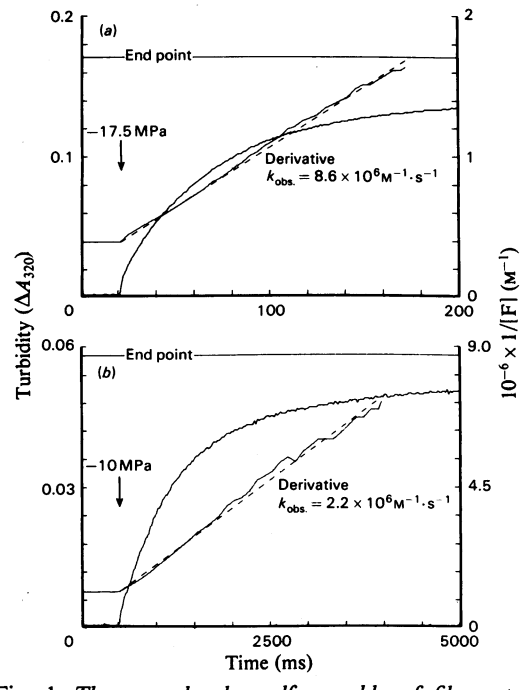

Fig. 1. *The second-order self-assembly of filament in the pressure-jump*

The relaxation was initiated by a step-wise (60μs) drop in pressure. The reaction was analysed in terms of a second-order reaction between like species. The total myosin concentrations used were (*a*) 2.5mg/ml and (*b*) 0.63mg/ml.

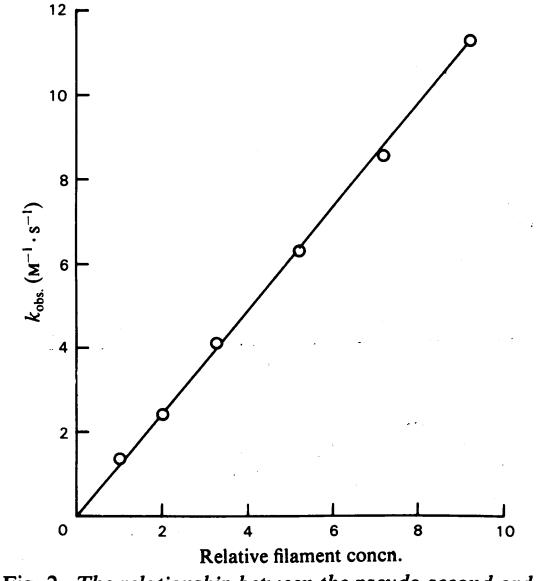

Fig. 2. *The relationship between the pseudo-second-order rate constant and filament concentration*

The rate constants were obtained from a series of relaxations similar to those depicted in Figs. 1(*a*) and 1(*b*). The graph passes through the origin, showing the assembly reaction to be third-order.

dissociation must not be so great as to allow the much slower process of filament nucleation to interfere with the propagation kinetics.

The relaxation curves for two different myosin concentrations are shown in Figs. 1(*a*) and 1(*b*). It can be clearly seen that the two second-order rate constants obtained are different. A direct relationship was shown to exist between the pseudo-second-order rate constants obtained and the turbidity of the solutions at atmospheric pressure. The turbidity of the myosin filament equilibrium at atmospheric pressure has previously been shown to be proportional to filament concentration (Davis, 1981). The relationship between the pseudo-second-order rate constant and filament concentration is made explicit in Fig. 2. A linear plot is obtained that passes through the origin of the graph; the observed second-order rate constant is thus directly proportional to the concentration of filament ends. The concentration dependence of the kinetics implies a third-order reaction mechanism of the following type where A represents the two like species that react and 2F the concentration of filament ends:

$$A + A + F_{i-2} \xrightarrow{k_{obs.}} F_i$$

$$\frac{d[F_{i-2}]}{dt} = -k_{obs.}[A]^2[2F]$$

The reaction is, however, unlikely to be termolecular, especially in the light of the recent discovery of a parallel dimer of myosin under identical experimental conditions (Davis *et al.*, 1981). All indica-

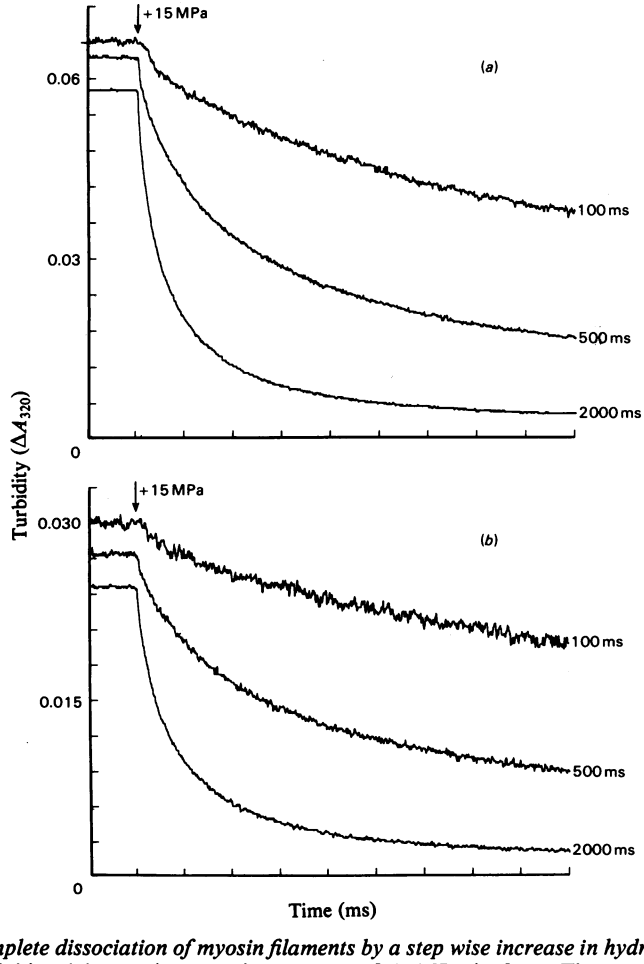

Fig. 3. *The complete dissociation of myosin filaments by a step wise increase in hydrostatic pressure*
Both relaxations were initiated by an increase in pressure of 15 MPa in 2 ms. The reactions were analysed by drawing tangents to the curves to obtain the velocity (pseudo-zero-order rate constant). The filament length was obtained from the percentage change in turbidity (see the text for details). The total myosin concentrations were (*a*) 0.6 mg/ml and (*b*) 0.3 mg/ml.

tions are that the dimer is the product of the reaction of the two like species seen in the self-assembly kinetics. The apparently termolecular reaction mechanism can thus be reduced to the two consecutive bimolecular reactions with monomer and dimer present in a rapid pre-equilibrium with each other before the rate-limiting addition of dimer (D) to the filament end. This mechanism and its relationship to the experimentally determined $k_{obs.}$ values is shown below:

$$M + M \overset{K_1}{\rightleftharpoons} D + F_{i-2} \xrightarrow{k_{+2}} F_i$$

$$\frac{d[F_{i-2}]}{dt} = k_{+2}K_1[M]^2[2F_{i-2}] = k_{obs.}[A]^2[2F]$$

A consequence of the proposed mechanism is that the rate of assembly of dimer on to the filament is independent of filament length.

Filament dissociation

A direct consequence of the rate of assembly of the thick filament from dimer being independent of filament length is that the co-operative reaction controlling filament growth has to be mediated through the dissociation reaction. This, together with the equilibrium data (Davis, 1981), would suggest that the narrow length distribution of the filaments could be established by a dissociation rate constant that increases with length until equilibrium is finally established. The length distribution of the filaments at equilibrium would as a result be proportional to the co-operativity of the dissociation reaction.

The kinetics were investigated by initiating the dissociation reaction with a stepwise (2 ms) increase in pressure to 15 MPa in the upward pressure-jump. The filaments were fully dissociated by the pressure used (Davis, 1981), thus reducing the effect of the reverse reaction on the kinetics to a minimum. It can quite clearly be seen in Figs. 3(*a*) and 3(*b*) that the relaxation profiles are non-linear and extend over a wide time scale. The dissociation rate appears to be fast at first, slowing markedly with progress of the reaction. Had the reaction been devoid of co-operativity the profile would have been linear up to a point where the filament concentration started to drop due to loss of filament. The high degree of co-operativity of the dissociation reaction is manifest in the temporal width of the relaxation spectrum.

A quantitative relationship between filament length and the dissociation rate constant can only be established for reactions showing either marked co-operativity or none at all. In a co-operative system a narrow filament-length distribution would be maintained by the 'sharpening' effect of a decreasing rate constant. This would result in the filament concentration remaining constant up to a point close to the end of the reaction when the shortest members of the length-distribution profile start to disappear. A tangent drawn to the reaction profile under these conditions gives the mean velocity or mean pseudo-zero-order rate constant for the dissociation reaction at the selected point in time. The mean filament length present at the point where the tangent was drawn can be determined from the turbidity of the solution (Davis, 1981). The turbidity at atmospheric pressure was taken to be equivalent to a 630 nm filament. The decrease in filament length from the initial 630 nm can be obtained from the percentage decrease in the turbidity of the solution at atmospheric pressure.

A linear relationship was found between the logarithm of the dissociation rate constant and filament length (Fig. 4). The data for the plot came from a series of tangents drawn to the reaction profiles shown in Fig. 3(*a*); the reaction at the lower total myosin concentration (Fig. 3*b*) exhibited parallel behaviour to that seen in Fig. 3(*a*). The pseudo-zero-order rate constant was found to decrease by a factor of 500 from the start to the finish of the dissociation reaction.

The only method available to test the validity of the assumptions used in the above analysis (Wiberg, 1974) is to simulate the reaction in the computer

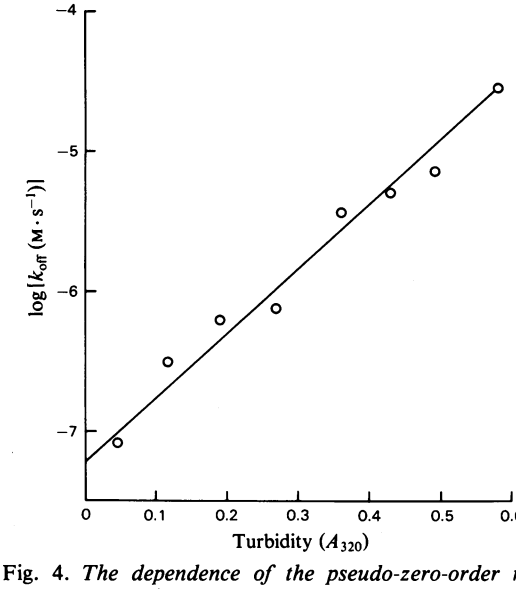

Fig. 4. *The dependence of the pseudo-zero-order rate constant for filament dissociation on filament length*
The data for the graph were obtained from Fig. 3(*a*). The rate of dissociation increases exponentially by a factor of 500 from left (the bare-zone length) to right (the 630 nm filament). The filament length is directly proportional to the turbidity scale (see the text).

using the experimental relationships obtained and to observe the molecular-weight distribution of the filaments as the reaction progresses. The number of myosin monomers packed into a 630nm filament can be calculated if one assumes a 3-fold symmetry for the filament (Squire, 1973) and a complete packing of monomer from the bare-zone out (Craig & Offer, 1976). 104 monomers are required; the value was rounded to 100 for convenience, which gave the synthetic filament a molecular weight of 46.5×10^6. The first-order rate-constant for the initial step in the dissociation reaction was calculated from the pseudo-zero-order rate constant and the filament molarity present in the reaction mixture. A first-order rate-constant of $1500\,s^{-1}$ was obtained for the initial step. A 50-step sequential reaction mechanism was set up with rate constants varying exponentially from $1500\,s^{-1}$ at the start to $3\,s^{-1}$ at the finish. The initial concentration of half-filaments was 40nM; each half-filament contained 50 monomer subunits. The amount of monomer liberated was used to follow the reaction.

The filament length-distribution present at 10%, 50% and 90% of full dissociation is shown in Fig. 5. A stable length distribution is set up, from the initially monodisperse 50-mer, at a very early stage in the reaction (10%), with the 50% and 90% length distributions being almost identical. The assumptions used for the kinetic analysis appear to be justified. As a result reliable measurements of the dependence of the mean rate constant for the dissociation reaction on mean filament length can be made safely over some 90% of the complete reaction profile. It seems reasonable to conclude that the length regulation mechanism is mediated through an ever-increasing dissociation constant that has an exponential dependence on filament length.

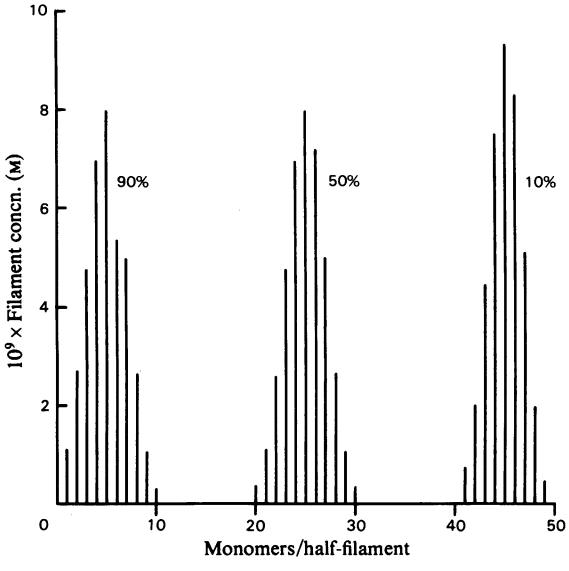

Fig. 5. *The filament-length distribution seen at different stages of the filament dissociation reaction*

A 50-step reaction was simulated with a first-order dissociation rate constant that decreased exponentially from $1500\,s^{-1}$ to $3\,s^{-1}$. The reaction was started with 50-monomer half-filament at a concentration of 40nM. It should be noted that a stable narrow filament length distribution is rapidly established, validating the kinetic procedure used to analyse the dissociation reactions of Figs. 3(*a*) and 3(*b*). The percentages in the Figure refer to dissociation.

Conclusion

The two kinetic mechanisms proposed for the association and dissociation reactions have some mechanistic and structural implications that extend beyond the basic sequence of events involved in each reaction. For example, the kinetic mechanisms can be used as a basis for speculation on the structure of the filament and on the relationship between the two mechanisms themselves. The studies also give additional support to some of the ideas put forward in the preceding paper (Davis, 1981).

There is a certain paradox in the relationship between the length-independent association and length-dependent dissociation reactions. Both reactions operate through sequential mechanisms, with the likely consequence of the one simply being the reverse of the other. In such a case, the dimer would have to add to the filament at the maximum rate for a diffusion-controlled reaction, facilitated in all probability by electrostatic interactions. A large $\Delta\bar{\nu}$ value for the reaction provides indirect evidence for the importance of charged groups in the assembly reaction (Davis & Gutfreund, 1976). Under the conditions described, the dimer would assemble at a rate independent of the length-dependent changes in the architecture of the binding site. The dissociation rate, on the other hand, would be controlled by the precise disposition of the amino acid residues at the binding site. A change in the alignment of these residues relative to the dimer structure would lead to a change in the affinity for dimer.

The control of filament length through the dissociation reaction has been shown to be mediated through an exponential increase in the 'off' rate with length. The increase in dissociation rate after each step occurs as a fixed percentage of the rate of that step. The precise physical mechanism whereby the high affinity, shown by the first pair of propagation sites at either side of the nucleus, declines uniformly will have to await more detailed kinetic and structural studies. The mechanism proposed should be

compatible with the mathematical relationship shown between length and the kinetic constants. There seems to be little doubt that the mechanism described plays an important role in establishing the specific structure of the thick filament.

The basic simplicity of the self-assembly kinetics has once again served to emphasize previous remarks (Davis, 1981) concerning the idea that it must be possible to build a model of the thick filament in a sequential and uniform manner from the bare-zone out. It would also be interesting to see whether any offset can be detected in the structure as it extends from the centre out.

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