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Tension responses to rapid pressure release in glycerinated rabbit muscle fibers

(force generation/phosphate release/actomyosin/crossbridge)

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ABSTRACT **We have previously shown that the isometric tension of a fully calcium-activated skinned rabbit psoas muscle fiber is reversibly depressed by increased hydrostatic pressure. We report here the characterization of tension transients induced by a rapid (<1-ms) release of increased pressure at 12°C. The tension transient consists of three clear phases, an initial further decrease of tension in phase with pressure change followed by two phases of tension increase back to the level recorded at ambient pressure. The mean reciprocal relaxation time for phase 2 ($1/\tau_2$) was $\approx 17\ s^{-1}$ and that for phase 3 ($1/\tau_3$) was $3\ s^{-1}$. The presence of 20 mM inorganic phosphate markedly increased $1/\tau_2$ to $\approx 52\ s^{-1}$ and decreased $1/\tau_3$ to $\approx 1.7\ s^{-1}$. These observations are interpreted in terms of a pressure-sensitive transition between two attached crossbridge states of low (or zero) and higher force. This is compatible with the pressure-sensitive isomerization of actomyosin previously observed in solution. The results presented allow us to propose a coupling between a specific pressure-sensitive isomerization of purified actomyosin, the phosphate release step of the ATPase pathway, and the force-generating event of the crossbridge cycle.**

One of the principal aims of research into the mechanism of force generation in muscle is to correlate the mechanical events of the crossbridge cycle with the biochemical events of the ATP hydrolysis cycle. The primary problem is that the mechanical events can be characterized only in a muscle fiber, while the biochemical events are most easily characterized in solutions of purified proteins. Few methods can readily be applied to both the purified proteins and those same proteins in the organized systems. Whereas rapid length and temperature perturbations have been successfully applied to contracting muscle fibers, these approaches have not been (and cannot be in the case of length changes) applied to the isolated proteins.

Changes in hydrostatic pressure have been used as a means to perturb the steady state of biological systems for more than 60 years, and contracting muscle was one of the first systems to be studied in detail (1–3). The interpretation of these early muscle studies was limited by the lack of information on the individual molecular components of the system. More recent work has demonstrated that pressure can be used to perturb protein–protein and protein–ligand interactions in solutions of purified components (4), and one of us has applied this approach to isolated muscle proteins (5).

In 1984 a two-step model was proposed to describe the binding of myosin subfragment 1 (S1) and S1·nucleotide complexes to actin in which an attached or A state is initially formed, followed by an isomerization to a tightly bound rigor-like or R state (6). The model was a development of the earlier work of Eisenberg and his collaborators (7, 8) but suggested that the ability to undergo the A-to-R (or weak to strong) transition was a general property of actomyosin·nucleotide complexes and not unique to one particular step of the ATPase pathway. Further, it was proposed that this A-to-R transition was coupled to the force-generating event in skeletal muscle. Since its proposal there have been several solution studies which have provided evidence in support of the model (reviewed in ref. 9). In particular it has been shown that increased hydrostatic pressure perturbs the A-to-R isomerization of the actin·S1 complex (5).

The solution model predicted that since the A-to-R transition is inhibited by pressure, then the development of isometric tension in a muscle fiber would be depressed by increased hydrostatic pressure; this has since been observed (10, 11). However, these studies did not establish that it was the A-to-R transition which was being perturbed in the contracting fiber.

MATERIALS AND METHODS

The tension transducer element is housed in a stainless steel tube, capped with an oil-filled glass tube. This then locates within a brass plug which itself inserts into the pressure chamber (10, 11). The pressure chamber was filled with experimental buffer solutions, details of which were given by Fortune *et al.* (11) (ionic strength 200 mM, pH 7.0, 12°C). Pressure was developed by using a hand-operated oil pump (Merlin, Preston, U.K.) and released by means of an in-line release tap ≈1 m away from the chamber (4). Pressure was monitored by means of a pressure transducer (Kistler 610A) located in the chamber and its signal at pressure release was used as a trigger for recording tension transients. Simultaneous records of pressure and tension were captured as 4000, 12-bit, data points, using a digital oscilloscope (Nicolet 3901). Each transient was transferred to a Hewlett–Packard 310 series computer and reduced to 400 data points for storage and subsequent analysis. Data were analyzed by using a nonlinear least-square fitting routine in HP Basic (Hi-Tech, Salisbury, U.K.).

All experiments were carried out on single muscle fibers from rabbit psoas muscle which were skinned and glycerinated (12). A fiber segment (≈4 mm) was mounted between two hooks on the tension transducer assembly (10) and was inserted into the pressure chamber, which was then filled with appropriate buffer and sealed for pressurization.

The experimental protocol for recording steady state tension responses at high pressure is given elsewhere (11). In the experiments presented here, a single isometric muscle fiber was pressurized and the tension response following rapid release of pressure was recorded. Typically, three or four individual tension responses were recorded under each experimental condition.

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Abbreviation: S1, myosin subfragment 1.

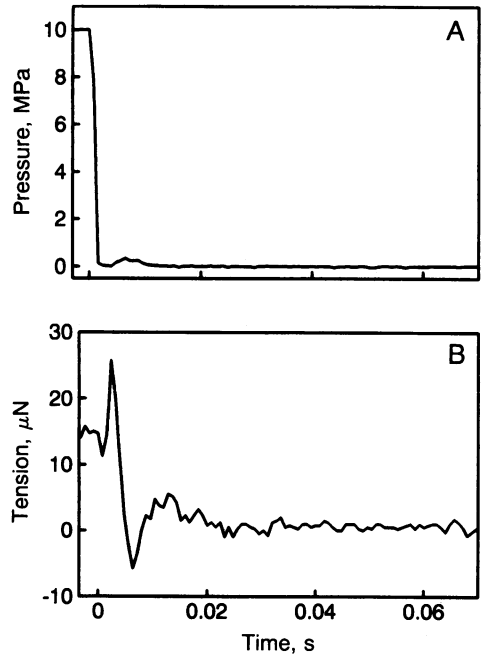

FIG. 1. Pressure (*A*) and tension (*B*) signals after a rapid pressure release with a relaxed single fiber mounted in the chamber (length 4 mm, diameter 80 μm). The pressure change was complete within 1 ms but the tension signal showed an oscillation over the first 4–6 ms (see text), after which the signal stabilized with a baseline shift of 15 μN.

Fig. 1*A* shows the time course of pressure release from 10 MPa back to ambient; the release of pressure is complete within 1 ms. The record also shows aftershocks in the pressure transducer output 4–6 ms after release. The tension response of a relaxed muscle fiber, routinely monitored in all experiments as a control, is shown in Fig. 1*B*. The record clearly shows there to be oscillations of the tension transducer over the first 4–6 ms after pressure release. The oscillations were found to be close to the natural resonant frequency of the tension transducer beam (2–5 kHz) when examined over a faster time scale (this is not apparent from Fig. 1*B* due to the collection of an insufficient number of data points). The time scale of the events within the first few milliseconds cannot therefore be accurately assessed. However, the tension signal stabilizes after this initial period, allowing any slower events to be followed. The tension transducer assembly both with and without an attached relaxed fiber normally showed a small signal offset on pressure release. The magnitude of this offset (≈15 μN in Fig. 1) was found to vary between individual transducers but to be both inherent and specific to individual elements. This offset was corrected for in data analysis; in Figs. 2 and 3 a horizontal arrow on the vertical axis indicates the corrected steady tension level before the pressure release.

RESULTS

Effect of Rapid Pressure Release on the Active Fiber. As reported previously, the steady active tension in a maximally Ca-activated fiber was depressed at high pressure. Fig. 2 shows the change in active tension after pressure release. It can be seen that there is a decrease of tension in phase with pressure release, followed by a recovery of tension back to the level observed before pressure was applied. The recovery of tension is shown in Fig. 2*A* fitted to a double exponential; the reciprocal relaxation times are 13.8 and 2.68 s^{-1}. The same trace is shown in Fig. 2*B*, with a single exponential fitted to the later part of the tension recovery, and in Fig. 2*C*

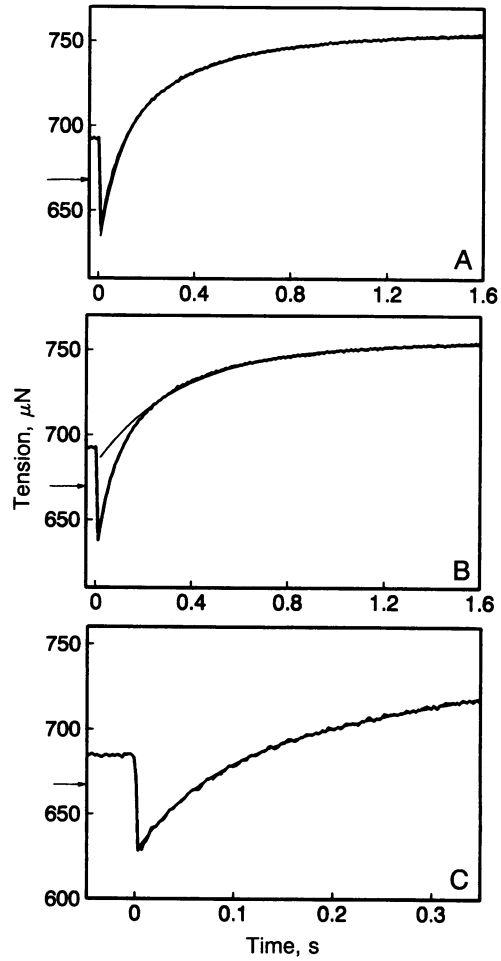

FIG. 2. Pressure-induced tension transients in a maximally Ca-activated muscle fiber. An observed transient is shown with best-fit double exponential (*A*) or single exponential over the later part of the transient (*B*) superimposed. The fit in *A* gives $1/\tau_2 = 13.8\ s^{-1}$ and $1/\tau_3 = 2.68\ s^{-1}$ and amplitudes of 61 and 64 μN, respectively; in *B*, $1/\tau_3 = 2.73\ s^{-1}$ and amplitude = 66 μN. (*C*) As in *A*, but with an expanded time base. A single exponential fit gives $1/\tau_2 = 13.9\ s^{-1}$ and an amplitude of 66 μN. Fiber diameter, 85 μm; length, 4 mm, steady-state active tension, 132 kN/m^2. In each case the arrow on the vertical axis indicates the steady tension at high pressure corrected to account for the transducer offset.

with a single exponential fitted to the first 400 ms of the trace. In both cases the data are well fitted by the single exponentials and the relaxation times are in good agreement with the double exponential fit. Thus, there appear to be two well-separated phases of tension recovery. The three components of the tension response (a rapid decrease of tension followed by two recovery phases) will be referred to as phases 1–3, respectively. Table 1 summarizes data from several experiments. The amplitudes of phases 1–3 were estimated by projecting the fitted line back to the time of pressure release.

Table 1. Characteristics of pressure-induced tension transients

Added P_i, mM	Phase	$1/\tau$, s^{-1}	*N*	*n*
0	2	16.88 ± 2.78	8	20
20	2	51.58 ± 11.5	4	8
0	3	3.07 ± 0.88	10	43
20	3	1.73 ± 0.82	8	25

Data show the mean (±SD) reciprocal relaxation times ($1/\tau$) for phases 2 and 3 in the presence and absence of 20 mM P_i. *n* gives the total number of measurements taken from *N* fibers.

From the double exponential fit shown in Fig. 2*A*, the amplitudes were −40, +61, and +63 μN, respectively, for phases 1, 2, and 3. This represents a decrease in initial tension of 5% for phase 1 and an increase of 8% for both phases 2 and 3. The net depression of steady tension at 10 MPa was approximately 12%. In separate experiments it was found that the rate at which the active tension recovered was independent of the magnitude of pressure used to perturb the system over the range 1–10 MPa. However, the amplitude of recovery was directly proportional to the pressure change (Table 2). The amplitude of phase 1 determined by extrapolation of the data in Fig. 2*A* (and given in Table 2) was relatively small. In a rigor fiber a release of pressure resulted in a decrease in tension in phase with the pressure change, followed by no observable tension recovery. These rigor results were interpreted in terms of a pressure-induced linear compression of an elastic element in series with the crossbridge (13).

Effect of Inorganic Phosphate (P_i) on Tension Recovery. In the presence of added P_i the pressure-induced transients show essentially the same features as in the absence of P_i but phase 2 becomes faster and phase 3 slower. The results from a single fiber are illustrated for 10 mM P_i in Fig. 3. Fig. 3*A* is shown with a single exponential fit to the slow phase of the tension recovery and gives $1/\tau$ of 2.4 s^{-1} for phase 3 in comparison to a control value (no added P_i) of 3.4 s^{-1} (not illustrated). A slow upward drift in tension was seen routinely in the presence of high concentrations of P_i after pressure release; such a marked drift was not seen in the control case. The data shown in Fig. 3*A* show a faster event complete in 40 ms (shown more clearly in Fig. 3*B*). A fit to the data gives $1/\tau$ of 55 s^{-1} for phase 2 in comparison with a control value of 18 s^{-1} (again not illustrated). As seen from Table 1, $1/\tau$ of phase 3 is reduced from 3 s^{-1} in the control to 1.7 s^{-1} in the presence of 20 mM added P_i; in contrast $1/\tau$ of phase 2 increased from a control value of ≈17 to 52 s^{-1} in the presence of 20 mM added P_i.

The phosphate concentration dependence of phases 2 and 3 was investigated, and results from one such experiment are shown in Fig. 4. As shown in Fig. 4*A*, $1/\tau_2$ increases with increasing P_i, and a hyperbolic fit to the data gives an apparent binding constant for phosphate of 3.92 mM and a maximum $1/\tau_2$ of 51 s^{-1}. The absolute amplitude of phase 2 decreased markedly with added P_i, while the normalized amplitude showed little change with increased P_i (Fig. 4 *B* and *C*). As shown in Fig. 4*D*, $1/\tau_3$ decreased from a control value of 2.35 ± 0.08 (control) to 1.09 ± 0.08 s^{-1} in the presence of 20 mM added P_i. The P_i dependence of the amplitude of phase 3 is shown in Fig. 4 *E* and *F* and indicates a decrease in absolute amplitude but a small increase in the amplitude when normalized to the steady active tension at atmospheric pressure.

DISCUSSION

The tension response to rapid release of pressure consists of three phases in the actively contracting fiber: an initial decrease of tension, complete within the pressure release time of the system, followed by two phases of recovery. The initial decrease was also seen in the rigor fiber, where isometric tension increased with hydrostatic pressure and decreased in phase with rapid pressure release. The underlying mechanical basis of the rigor response has been discussed in detail and indicates normal (nonrubberlike) elasticity (13). Since isometric tension in a stretched relaxed fiber (passive tension) showed no pressure sensitivity, the response of the rigor fiber must involve perturbation of an elastic element that does not contribute to passive tension and is, therefore, introduced by the rigor crossbridge. The normal elastic mechanical behavior is also present transiently in the active fiber where the crossbridges are cycling.

A rapid length release also leads to a decrease of tension in both active and rigor muscle fibers (14, 15), representing the response of series elastic elements in the crossbridge. The experiments of Ford *et al.* (14, 15) showed that in the case of the active fiber the tension recovered (T1–T2 transition) following the rapid tension decrease. The linear compression equivalent to a pressure rise was determined previously for rigor fibers and was 0.3% for 10 MPa (13). In an active fiber a 0.4–0.6% length release will reduce the tension to zero (14). Therefore, if the compression induced by 10 MPa of pressure in the active fiber is similar to that observed in rigor, a rapid

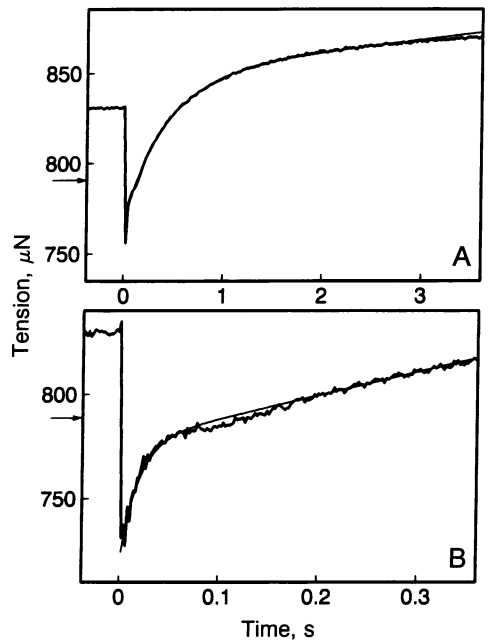

FIG. 3. Tension transients in the presence of 10 mM P_i. (*A*) A single exponential fit to the later part of the tension transient giving $1/\tau_3$ of 2.32 s^{-1} and an amplitude of 81 μN. (*B*) A second pressure-induced tension transient on the same fiber as in *A* with faster data collection, showing the early part of tension transient. A single exponential fit with a sloping baseline gives $1/\tau$ of 55 s^{-1} for phase 2 and an amplitude of 35 μN. The arrow on the vertical axis indicates the steady tension at high pressure corrected to account for the transducer offset. The steady tension was 90.3 kN/m^2 in the presence of 10 mM added P_i.

Table 2. Pressure dependence of tension transients

Pressure, MPa	Phase 1 amplitude, %	Phase 2		Phase 3	
		$1/\tau_2$, s^{-1}	Amplitude, %	$1/\tau_3$, s^{-1}	Amplitude, %
10	4.18 ± 1.61	17.3 ± 3.63	11.35 ± 0.35	1.83 ± 0.136	14.75 ± 0.46
6	1.92 ± 0.122	16.3 ± 2.12	6.40 ± 0.93	1.34 ± 0.07	9.02 ± 0.08
3	—	17.4 ± 3.03	3.00 ± 0.91	1.38 ± 0.26	4.66 ± 0.63

Data are for a single fiber at 12°C; amplitudes are expressed as a percentage of the isometric tension at atmospheric pressure in each case. Each value is the mean (±SD) of four or five individual observations. No data for the amplitude of phase 1 are available for 3 MPa, as the change was within the signal noise of the transducer.

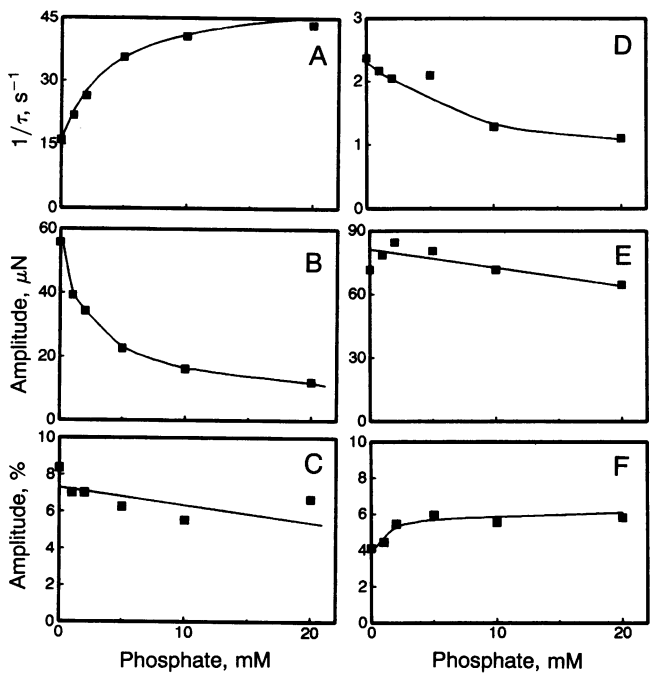

FIG. 4. Phosphate dependence of phases 2 and 3 of tension recovery. The P_i dependence of the reciprocal relaxation time (*A*, *D*), the amplitude (*B*, *E*), and the percentage amplitude (*C*, *F*) of phase 2 (*A–C*) and phase 3 (*D–F*). Fiber characteristics: for *A–C*, diameter 85 μm, length 4 mm, initial tension in the absence of added P_i 141 kN/m^2; for *D–F*, diameter 75 μm, length 4 mm; initial tension in the absence of P_i 113 kN/m^2. All the lines are drawn by eye except in *A*, where the fitted hyperbola gives an apparent binding constant for phosphate of 3.92 mM and a maximum reciprocal relaxation time of 51 s^{-1}.

transient decrease in tension of approximately 50% would be expected on pressure release. However a much smaller tension decrease is routinely observed (≈4%, Table 2). If phase 1 of the pressure-induced tension transient is equivalent to a length change after release of pressure, then a rapid recovery, similar to the T1–T2 transition, is also expected. As the tension measurements over the first 4–6 ms are lost in transducer oscillations we cannot eliminate the possibility that our phase 1 consists of a much larger (≈50%) tension fall followed by a rapid T1–T2 type recovery almost complete within 4–6 ms. Faster pressure releases with a more stable transducer or use of slower muscle fibers may be required to identify such processes. An instantaneous tension decrease is also seen in response to a small temperature jump in both rigor and active fibers (16–18).

The early tension decrease is followed by two clear phases of tension recovery that are absent in the rigor fiber (13) and therefore reflect the response of the cycling crossbridges in the active fiber to the pressure change. The form of the recovery of tension appears to rule out dissociation of actin or myosin from the filaments. Support for this idea comes from equatorial x-ray diffraction studies on skinned fibers at high pressure (19), which show no measurable change in the position or intensity of the equatorial reflections from a small bundle of relaxed, active, or rigor muscle fiber. This suggests that the structural basis of the observed reduction of active tension at high pressure is a result of a loss of force per crossbridge rather than a reduction in the number of operating crossbridges. The first recovery event observed after pressure release (phase 2) is much faster than the ATP turnover rate and therefore represents a pressure-sensitive transition from a low-force to higher-force state (or states) occurring before the rate-limiting step of the ATPase pathway. The slower tension recovery event, phase 3 (approximately 3 s^{-1}), occurs at a rate close to that of the ATPase turnover rate (20–22). It therefore represents a population of crossbridges that pass through the rate-limiting step in the crossbridge cycle before being able to contribute again to tension development. This slower relaxation could be either a second pressure-sensitive event or an event coupled to the first pressure-sensitive transition.

Phases 2 and 3 fully account for the recovery of pressure-induced depression of active tension, and they are considerably slower than the force recovery event observed in length release experiments (14). They also differ from the phases observed after a temperature jump (16–18). The application of different perturbation methods to a system may perturb different physicochemical events. The perturbation of different steps of the crossbridge cycle and analysis of the mechanical response following such a perturbation will lead to a better understanding of the relationship between mechanical and biochemical events in the cycle. This is particularly true if the identity of the biochemical event that is perturbed is known.

A pressure-sensitive isomerization of both actin·S1 and actin·S1·nucleotide complexes has been identified in solution (the A-to-R transition; see *Introduction*). The simplest interpretation of the pressure-induced transients is therefore that the A-to-R transition is the event that is perturbed and is responsible for phase 2 of the tension recovery. In support of this argument, related work has shown that in solution nucleotide binding to S1, nucleotide binding to actin·S1, and the cleavage of ATP on myosin occur with no significant pressure sensitivity (23). This interpretation is also consistent with the ATPase model of Geeves *et al.* (6), which originally proposed that the A-to-R transition could be coupled to the force-generating event of the crossbridge cycle (7).

In this interpretation phase 2 represents the relaxation of crossbridges from the A to R state in the crossbridge cycle.

$$A \rightleftharpoons R \rightarrow \rightarrow \rightarrow \text{detached} \rightarrow \rightarrow \rightarrow A$$

where the A-to-R transition is pressure sensitive, and the A and R states are low- and high-force crossbridge states, respectively. The arrows indicate an unknown number of additional steps, including the step responsible for phase 3.

In the muscle fiber changes in the concentration of free ADP and P_i will affect the population of crossbridges in the steady state (24) and, therefore, change the properties of the transition involved in phase 2.

The P_i dependence of phase 2 shows the classical behavior of a two-step ligand-binding event

$$\underset{}{A{\cdot}P_i} \underset{1}{\rightleftharpoons} R{\cdot}P_i \underset{2}{\rightleftharpoons} R + P_i$$

where the P_i-binding step is a rapid equilibrium and the A-to-R step, as above, is pressure sensitive. In this case the observed relaxation is defined by

$$1/\tau_2 = \frac{k_{-1} \cdot [P_i]}{K_2 + [P_i]} + k_{-1}.$$

Thus K_2 = 3.9 mM and $k_{+1} + k_{-1}$ = 51 s^{-1}.

The coupling of the phosphate release step to the force-generating transition has been a long-standing feature of the mechanochemical cycle based on solution studies (7, 25) and more recently supported by fiber studies (26, 27). Isotope-exchange studies have demonstrated that P_i binds to the myosin active site during an isometric contraction and equilibrates with the P_i in myosin-bound ATP (20). Experiments using caged P_i (a photolabile precursor of P_i) have shown that P_i binds reversibly to force-bearing crossbridges and that binding of P_i results in loss of tension from the crossbridge

(28). Interestingly, the concentration dependence of the rate of force decline is not linear but approaches a maximum observed rate, suggesting a similar two-step process for P_i release/binding (where $K_2 = 13$ mM and $k_{+1} + k_{-1} = 130\ s^{-1}$ at 10°C and ionic strength = 0.2 M). Thus, although the P_i dependence of the relaxation time of phase 2 on release of pressure is not identical to that of the caged P_i experiment, there is a similarity in the concentration dependence of the two perturbations.

The solution model predicts that both the A and R states in the contracting muscle correspond to more than one actin·S1·nucleotide state and that the properties of the A-to-R transition will depend upon the nature of the nucleotide bound. For example, the equilibrium constant for the transition in solution changes from $>10^2$ in the absence of nucleotide to $\leq 10^{-2}$ when ATP is bound even though the rate of the transition remains $>2000\ s^{-1}$ in both cases. With ADP bound the equilibrium constant is ≈ 10 and the rate is $\approx 5\ s^{-1}$ (9). These numbers cannot be readily translated to fibers, as they will be strain dependent if the transition is coupled to the force-generating event.

In summary, the work presented here demonstrates that a pressure-sensitive transition can be identified in a contracting muscle fiber that is coupled to force generation, and its properties are compatible with those of the protein isomerization identified in solution. Furthermore, we demonstrate that the pressure-sensitive isomerization of the crossbridge proteins is coupled to the P_i release step in the crossbridge cycle. The nature of the coupling at the molecular level remains unresolved; in the model of Geeves *et al.* the role of P_i release is to trap the actomyosin in the tension-holding R state. Whether the A-to-R transition itself is the molecular event that generates the strain between the two filaments or whether formation of the R state allows a mechanical transition (such as the T1–T2 transition of Ford *et al.*, or helix-coil melting) to occur cannot be distinguished on the data presented here.

Finally, we have demonstrated the advantage of using a perturbation technique that can be applied both to the purified proteins and to the organized biological system containing the same proteins. Pressure has been shown to perturb many protein–protein interactions in solution that are central to the organization and motile activity of single cells [microtubule assembly, actin filament assembly, myosin filament assembly, and G-protein interactions (29–31)]. Earlier work had already shown that motile behavior of single cells can be reversibly inhibited by high pressure (3), and therefore the approach outlined here may be used to advantage in these systems.

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