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Indirect coupling of phosphate release to *de novo* tension generation during muscle contraction

(laser temperature jump/relaxation kinetics/thermodynamics/stretch activation/skinned rabbit fibers)

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ABSTRACT **A key question in muscle contraction is how tension generation is coupled to the chemistry of the actomyosin ATPase. Biochemical and mechanochemical experiments link tension generation to a change in structure associated with phosphate release. Length-jump and temperature-jump experiments, on the other hand, implicate phase 2_{slow}, a significantly faster, markedly strain-sensitive kinetic process in tension generation. We use a laser temperature jump to probe the kinetics and mechanism of tension generation in skinned rabbit psoas fibers—an appropriate method since both phosphate release and phase 2_{slow} are readily perturbed by temperature. Kinetics characteristic of the structural change associated with phosphate release are observed only when phosphate is added to fibers. When present, it causes a reduction in fiber tension; otherwise, no force is generated when it is perturbed. We therefore exclude this step from tension generation. The kinetics of *de novo* tension generation by the temperature-jump equivalent of phase 2_{slow} appear unaffected by phosphate binding. We therefore propose that phosphate release is indirectly coupled to *de novo* tension generation via a steady-state flux through an irreversible step. We conclude that tension generation occurs in the absence of chemical change as the result of an entropy-driven transition between strongly bound crossbridges in the actomyosin–ADP state. The mechanism resembles the operation of a clock, with phosphate release providing the energy to tension the spring, and the irreversible step functions as the escapement mechanism, which is followed in turn by tension generation as the movement of the hands.**

Energy transduction in muscle is mediated by a complex sequence of biochemical and mechanical intermediates that form and decay during the crossbridge cycle. Consequently, change(s) in structure specific to energy transduction has to be resolved against a background of conformational changes typical of the normal function of an ATPase enzyme transiently activated by complex formation with a second protein. Of the biochemical steps, phosphate release from the active site of myosin (1–4), with its associated large free energy change (5) (see reviews in ref. 6–8), has received considerable attention in relation to tension generation. An isomerization (structural change) in the crossbridge immediately before phosphate release from the active site of myosin has emerged as the single step most likely associated with tension generation.

As yet, no plausible connection has been made between the biochemistry and tension recovery in small length-jump/step (L-jump) experiments. In the classical Huxley–Simmons L-jump experiments, the large amplitude fast recovery of tension (phase 2) immediately following the length change has traditionally been associated with tension generation. A slower, phosphate-sensitive component of the L-jump kinetics (phase 3) has also received attention in the quest to link phosphate release to a mechanical step (9–11). However, this step fails to generate tension when perturbed (12). The lack of correspondence between the biochemical and mechanical steps introduces the possibility of indirect, rather than direct, coupling between steps.

We use a laser temperature jump (T-jump) to very rapidly heat a contracting muscle fiber a few degrees in order to explore the linkage between phosphate binding and the mechanical step we believe to be the *de novo* tension-generating step (12–14). The method is particularly appropriate because both the main biochemical step [the isomerization preceding phosphate release (3, 11)] and the mechanical step linked to *de novo* tension generation [phase 2_{slow} (12–14)] show marked temperature sensitivity. In addition, both steps have equilibrium constants close to unity so that a change in temperature will cause the largest possible change in the concentrations of the reactants and thus tension (3, 11). To date, laser T-jump experiments have failed to elicit a response with the kinetic characteristics of the isomerization that precedes phosphate release (12, 15). The T-jump equivalent [medium speed relaxation $(1/\tau_2)$] of the mechanical phase 2_{slow} was, however, present (12–14). These data indicate that *de novo* tension generation is probably associated with the mechanical step and not with the isomerization that precedes phosphate release.

In this paper, laser T-jump experiments are performed under conditions where phosphate release is readily reversible to determine its relationship to tension generation. The results are straightforward: phosphate (15 mM) introduces a new kinetic component (relaxation) that serves to depress, rather than increase, tension. This is consistent with the general observation that the isomerization preceding phosphate release does not generate tension (12). The T-jump equivalent of the mechanical phase 2_{slow} that is associated with *de novo* tension generation shows no sign of linkage to the markedly temperature-sensitive binding of phosphate to the myosin active site. In contrast, phosphate binding must be linked in the forward direction to *de novo* tension generation because perturbation of the phosphate binding equilibrium in the T-jump causes a drop in fiber tension. We propose a plausible mechanism, consistent with these observations, in which the group of equilibria (strictly speaking, steady-state intermediates in quasi-equilibrium) associated with phosphate binding are indirectly coupled to the tension-generating equilibrium by changes in the steady-state flux through an irreversible step. A preliminary report describing these experiments has been published (16).

MATERIALS AND METHODS

The standard activating solution contained 7.39 mM $MgCl_2$, 5.52 mM vanadium-free ATP, 20 mM CaEGTA, 20 mM creatine phosphate, 15 mM disodium glycerol 2-phosphate,

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Abbreviations: L-jump, length jump; T-jump, laser temperature jump; P-jump, pressure jump; P_i-jump, phosphate jump.

and 2 mg of creatine phosphokinase per ml adjusted to pH 7.1 at room temperature (15). Phosphate was added to relaxing, preactivating, and activating solutions by substituting the 15 mM disodium glycerol 2-phosphate buffer with 15 mM disodium phosphate. This maintained the 0.2 M ionic strength of the solutions and the requisite temperature insensitivity of the buffer; compositions of the relaxing and preactivating solutions used are described elsewhere (13).

Single, chemically skinned rabbit psoas fibers were prepared and mounted on the tension transducer with aluminum T clips (13). In L-jump experiments, at low to moderate temperatures, these fibers typically require releases of between −5 and −8 nm per half sarcomere to reduce tension of the extrapolated T_1 curve to zero (y_0). Reliability of our small-perturbation kinetic data over the full temperature range used is discussed in detail elsewhere (12, 13).

Details of the design, construction, and use of the infrared iodine photodissociation laser and its associated optics used to heat the muscle fiber uniformly in <1 μs are described elsewhere (13, 17). A model 407A (Cambridge Technology, Watertown, MA) capacitor-based force transducer with a resonant frequency of 12 kHz, a 100-μs rise time, and a compliance of 0.1 $\mu m \cdot g^{-1}$ was used for all tension measurements. Voltage output from the force transducer amplifier was recorded on a digital oscilloscope (model 430; Nicolet) as variable length records (generally 8000 data points) of 12-bit data. An in-house program using LabView software running on a Gateway 2000 4DX2-66V computer with an AT-GPIB interface card (National Instruments, Austin, TX) was used to control data acquisition. Details of the nonlinear least-squares method used for data analysis remain unchanged (13).

RESULTS AND DISCUSSION

Small-perturbation or classical relaxation kinetic theory is used to analyze the kinetics. A T-jump of a fiber simultaneously perturbs all temperature-sensitive steps in the cycle a small amount. The observed tension transient arises from the kinetically controlled adjustment of the steady state to new conditions at the higher temperature and has the typical form of the sum of a series of exponential processes (or relaxations).

The experiments we present are specifically designed to determine the relative location in the crossbridge cycle of two temperature-sensitive equilibria—one associated with phosphate release, the other associated with *de novo* tension generation. Maximally Ca^{2+}-activated muscle fibers contracting isometrically are used in the experiments (12–14, 18). Under these conditions of steady-state ATP hydrolysis the mechanochemical cycle can be subdivided into groups of steps close to equilibrium separated by irreversible or rate-limiting steps. The rules are straightforward: Two kinetic steps are in the same group of reactions at equilibrium if they are kinetically coupled (occur on the same time scale) or equilibrium coupled (the fast step equilibrates before the slow step) to one another. They are in separate groups if they appear linked in one direction only via the steady-state flux through an irreversible step.

Effect of Phosphate on T-Jump Kinetics. In the absence of added phosphate, three relaxations ($1/\tau_1$, $1/\tau_2$, and $1/\tau_3$), all with positive amplitudes, govern the increase in tension following a T-jump in fully activated muscle fibers; addition of 15 mM phosphate depresses isometric tension and introduces a fourth relaxation with a distinctive negative amplitude (Fig. 1). This is consistent with the observation that phosphate both depresses isometric tension (1, 2, 19) and binds tighter at higher temperatures (3, 11). We term this T-jump relaxation $1/\tau_{negative}$. It is generally below the limits of detection in experiments without added phosphate despite the presence of low concentrations of endogenous phosphate in the fiber. We do detect it on occasion with batches of creatine phosphate that have inorganic phosphate as contaminant. The various fitted exponential components that sum to generate the tension transients are illustrated in Fig. 1 *B* and *D* and are described in the legend. A series of tension transients recorded at different temperatures in the presence of phosphate are illustrated in Fig. 2. A small, very slow linear component, similar to that seen in pressure-jump (P-jump) (20) and phosphate-jump (P_i-jump) (3) experiments, is present on occasion. $1/\tau_{negative}$ with its characteristic negative amplitude

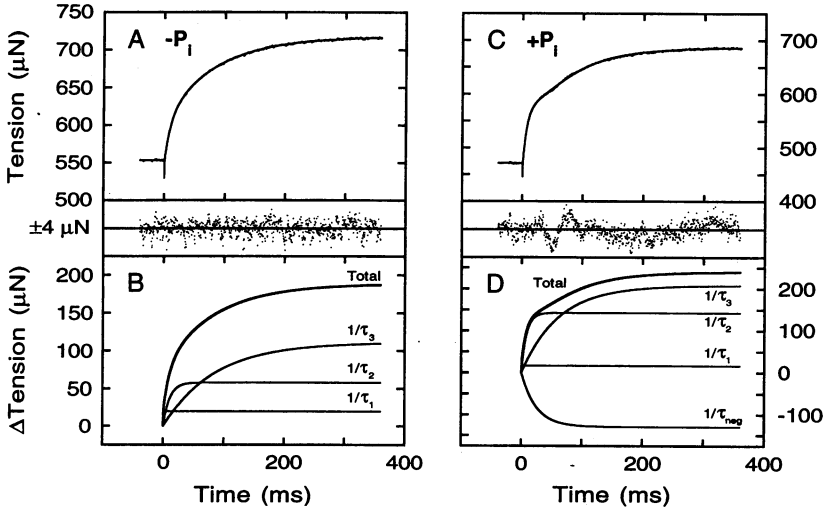

FIG. 1. Laser T-jump tension transients in the presence and absence of added phosphate. In the absence of added phosphate (*A* and *B*), the increase in isometric tension on heating is fully accounted for by the slow temperature-insensitive relaxation ($1/\tau_3$) and the medium speed, temperature-sensitive relaxation ($1/\tau_2$) (14, 15, 18). We exclude the fastest relaxation ($1/\tau_1$) from discussion because it arises from a mini L-jump imposed on the fiber by the step expansion of the proteins following the T-jump (13, 15). Addition of 15 mM phosphate (*C* and *D*) depresses isometric tension from 125.3 to 72.7 kN/m^2 and introduces another relaxation, $1/\tau_{negative}$, with a negative amplitude. Kinetic components simulated from the fitted parameters are plotted in *B* and *D*. Residuals plotted below transients *A* and *C* were obtained by subtracting the fitted line from the experimental data. The laser T-jump heated the fiber by 5°C in <1 μs to a postjump temperature of 11°C. The relaxed sarcomere spacing was 2.5 μm. The fitted rate constants and amplitudes were $1/\tau_1 = 3449\ s^{-1}$, $a_1 = 19.7\ \mu N$, $1/\tau_2 = 94.8\ s^{-1}$, $a_2 = 57.6\ \mu N$, $1/\tau_3 = 11.8\ s^{-1}$, and $a_3 = 111.2\ \mu N$ without added phosphate and $1/\tau_1 = 2694\ s^{-1}$, $a_1 = 16.9\ \mu N$, $1/\tau_2 = 121\ s^{-1}$, $a_2 = 142.6\ \mu N$, $1/\tau_3 = 16.4\ s^{-1}$, $a_3 = 208.3\ \mu N$, $1/\tau_{negative} = 39.9\ s^{-1}$, and $a_{negative} = -129.2\ \mu N$ with added phosphate.

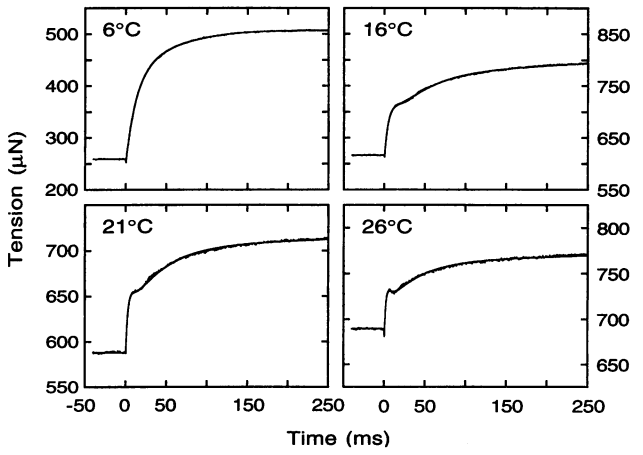

FIG. 2. Effect of temperature on laser T-jump kinetics in the presence of added phosphate. Note the increase in rate of the phosphate-dependent $1/\tau_{negative}$ from where it overlaps the slow relaxation ($1/\tau_3$) at 6°C to where it comes close in value to the medium-speed relaxation ($1/\tau_2$) at 26°C. Postjump temperatures are indicated. Other conditions are similar to those in Fig. 1.

is apparent at all temperatures save at 6°C, where its rate is coincident with the slow relaxation ($1/\tau_3$).

Temperature Dependencies of the T-Jump Kinetic Phases. Plots of the temperature dependencies of the various relaxations (Figs. 3–5) are used to determine whether a particular kinetic step is coupled to phosphate release. Coupling is indicated by a phosphate-induced change in the apparent rate constant for the step that should, in addition, vary with temperature, since phosphate binding is markedly temperature sensitive (3, 11).

The slow relaxation ($1/\tau_3$) is unlikely to generate tension because it is too slow, is insensitive to temperature, and is thought to arise from a change in steady-state flux through a rate-limiting step (12, 13). Its incorporation into a basic three-step mechanism of contraction that includes a rapid preequilibrium and the medium speed relaxation ($1/\tau_2$) is described in detail elsewhere (12). Added phosphate results in a small (≈55%) increase in its rate regardless of temperature (Fig. 3). This observation is consistent with the equally small increase in the apparent rate constants of the related L-jump phase 4_b (12) and k_{ADP} [the rate constant that governs the tension increase following the step release of caged ADP in

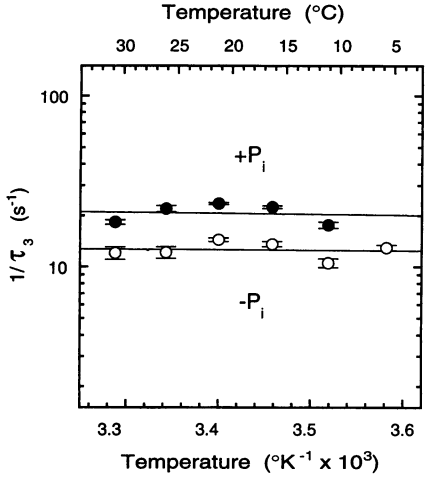

FIG. 3. Effect of phosphate on temperature dependence of $1/\tau_3$, the slow T-jump relaxation. The relaxation is probably the equivalent of the L-jump phase 4_b (12). Error bars indicate ±SEM. At 6°C, $1/\tau_3$ and $1/\tau_{negative}$ are too close in rate to be resolved.

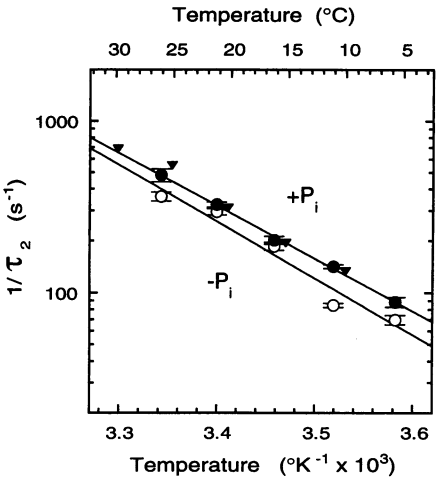

FIG. 4. Effect of phosphate on the temperature dependence of $1/\tau_2$, the medium-speed T-jump relaxation; 15 mM added phosphate has virtually no effect on rate. The relaxation is associated with *de novo* tension generation and is equivalent to the L-jump phase 2_{slow} (12–14). We illustrate this by superimposing replotted phase 2_{slow} data from Zhao and Kawai (11) (▼) obtained at a similar phosphate concentration (8 mM) using sinusoidal length oscillations that, unlike our own L-jump phase 2_{slow} data (12, 13), are strain neutral. Error bars indicate ±SEM. At 31°C, $1/\tau_2$ and $1/\tau_{negative}$ are too close in rate to be resolved.

fibers (21)] in the presence of similar concentrations of phosphate.

The medium-speed relaxation ($1/\tau_2$) is the equivalent of the L-jump phase 2_{slow} and is thought to arise from a transition between the pre-force-generating and force-generating states (12–14). The following observations indicate that phosphate binding and release are kinetically isolated and uncoupled from $1/\tau_2$. First, a small (≈30%) increase in $1/\tau_2$ is caused by the addition of 15 mM phosphate at all temperatures (Fig. 4)—a minor change that is clearly unrelated to the ≈300% increase seen in k_{P_i} in P_i-jump experiments over a similar change in phosphate concentration (3). Second, the temperature-independent change in $1/\tau_2$ with added phosphate is

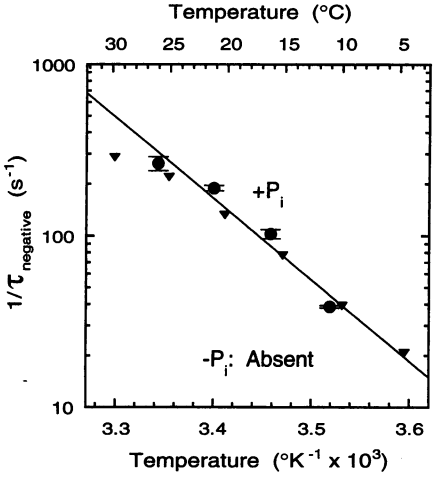

FIG. 5. Temperature dependence of $1/\tau_{negative}$, the phosphate-dependent T-jump relaxation. This relaxation is not observed in the absence of added phosphate. Replotted data from Zhao and Kawai (11) (▼) for the L-jump phase 3 at the same phosphate concentration superimpose on our T-jump data. This establishes a common mechanistic origin for both relaxations. Error bars indicate ±SEM. $1/\tau_3$ and $1/\tau_{negative}$ at 6°C and $1/\tau_2$ and $1/\tau_{negative}$ at 31°C are too close in rate to be resolved.

quite distinct from the marked increase in the affinity of myosin for phosphate seen in contracting fibers as temperature is raised (3, 11). Furthermore, the rate of the related L-jump phase 2_{slow} (phase b and thus phase 2_{slow}) (22) is equally little affected by phosphate.

The new phosphate-induced T-jump relaxation ($1/\tau_{negative}$) is present at all temperatures studied (Fig. 5). Its negative amplitude serves to partly diminish the increase in tension arising from $1/\tau_3$ and $1/\tau_2$ (Figs. 1 *C* and *D* and 2). The fact that our $1/\tau_{negative}$ rate vs. temperature data and similar L-jump kinetic data for phase 3 (11), obtained at the same phosphate concentration, superimpose (see Fig. 5) shows that the two kinetic processes are the same. We earlier concluded that phase 3 does not generate tension on the basis of L-jump and T-jump experiments performed in the absence of added phosphate (12). The negative amplitude of $1/\tau_{negative}$ in the T-jump in the presence of phosphate further substantiates this interpretation. Previously, phase 3 was thought to be associated with crossbridge detachment (23–25).

Mechanism for Indirect Coupling of Phosphate Release to Tension Generation. We interpret and unify our experimental observations according to the scheme illustrated and described in Fig. 6. Step 1 includes phosphate release and the isomerization that precedes it. Force is not generated during this step because our T-jump experiments rule our *de novo* tension generation by the related $1/\tau_{negative}$, phase 3, k_{P_i} and phase 2 in T-jump, L-jump, P_i-jump, and P-jump (20) experiments,

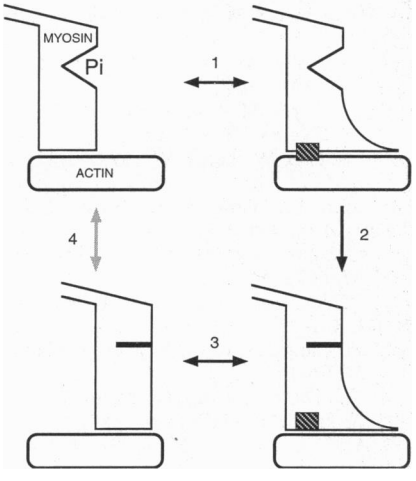

FIG. 6. Schematic representation of relationship between tension generation and phosphate release during muscle contraction. Phosphate release and the isomerization that precedes it occur between strongly bound states in step 1. Isomerization is readily reversible and easily perturbed; reversibility of phosphate release depends on phosphate concentration. The large free energy change associated with step 1 is stored in the structure of the crossbridge, but the state is "locked" and cannot generate tension. Step 2 is an irreversible conformational change between actomyosin–ADP states that serves the dual purpose of releasing the lock on tension generation and closing the phosphate binding site. *De novo* tension generation occurs in step 3 with the interconversion of the pre-tension-generating state to the tension-generating state. The steady-state flux through the irreversible step 2 couples phosphate release (step 1) to tension generation (step 3) in the forward direction but effectively prevents reverse coupling of tension generation to phosphate release. Step 4 completes the crossbridge cycle and includes a number of steps such as ADP release, ATP binding, crossbridge detachment and reattachment, and ATP hydrolysis that ultimately lead to formation of the illustrated strongly bound actomyosin–$ADPP_i$ state. The slow T-jump relaxation $1/\tau_3$ and L-jump phase 4_b correlate with step 4. The medium speed relaxation $1/\tau_2$ and L-jump phase 2_{slow} correlate with step 3. The phosphate-induced T-jump relaxation $1/\tau_{negative}$ and L-jump phase 3 arise from perturbation of step 1 coupled via the steady-state flux through step 2 to tension generation in step 3.

respectively. Tension changes recorded with the kinetics of these related phases arise indirectly by a partial reversal of step 1 and possibly elements of step 4 and a concomitant reduction in the steady-state flux through step 2. This drop in flux reduces the reactant concentrations participating in tension generation in step 3 and thus diminishes tension. The presence of strongly bound crossbridges that generate no tension and function in step 1 is indicated by the fact that fiber stiffness declines less than force when phosphate is added in kinetic (3, 26) or steady-state experiments (22, 27).

The kinetics of *de novo* tension generation (step 3) associated with the T-jump $1/\tau_2$ or L-jump phase 2_{slow} appear isolated from the temperature-dependent binding of phosphate to the active site of myosin described above. We therefore postulate that tension generation must occur after phosphate release and be isolated from it. The irreversible transition of step 2 in Fig. 6 serves the essential role of isolating the force-generating equilibrium from equilibria associated with phosphate binding and release. Steps associated with phosphate release control tension generation by modulating the flux of crossbridges through step 2. Step 4 completes the cycle.

We were struck by a possible relationship between our kinetic scheme and the thesis that myosin might function as a "back door" enzyme, an idea proposed in a paper on the structure of the myosin head (28) that appeared during the review of this work. The suggestion is that phosphate might leave myosin not from its entry point in the nucleotide binding cleft but via the 50-kDa cleft, which then closes shut. Closure of the 50-kDa cleft after phosphate release may correspond to step 2 in our scheme, where it functions to both prevent phosphate rebinding and remove the lock on tension generation.

Stretch Activation and the L-Jump Response. In our model, phosphate binding is indirectly coupled to tension generation by the steady-state flux. Perturbation of the equilibria associated with phosphate release causes tension to change with the kinetics of the L-jump phase 3. Partial reversal of these equilibria in P_i-jump (by added phosphate), in T-jump (by increased phosphate affinity), and in L-jump step-release (by strain-induced crossbridge dissociation) experiments causes tension to decrease. A forward bias of these equilibria in P-jump experiments (by increased actomyosin affinity) causes tension to increase (20). Accordingly, an L-jump step stretch is expected to strain attached crossbridges and cause a drop in tension. The opposite is observed and tension rises with the kinetics of phase 3. A plausible explanation is that an increase in the number of actin-attached crossbridges, in response to stretch, mediates the response. Force enhancement (above isometric tension) during steady lengthening of vertebrate muscle fibers results, in part, from an increase in the number of actin-attached crossbridges (29). If the phase-3 response similarly arises from crossbridge recruitment during stretch, it should be suppressed when a step stretch is applied to fibers during continuous extension. This has been observed experimentally (29). Stretch activation, a much more dramatic response, seen in insect flight muscle also exhibits phase-3 kinetics. A possible steric origin for the enhancement of the phase-3 response is indicated by the fact that the thick- and thin-filament subunit repeats match in insect flight muscle (30), and the tension increase is reduced in vertebrate muscle, where the subunit repeats do not match. We conclude that step 1 of our scheme could have the important regulatory role of indirectly modulating the number of tension-generating crossbridges during muscle contraction.

Mechanistic Implications of the Rate of Phase 2 Recovery Following an L-Jump Step Release. A full functioning of the characteristic features of the scheme in Fig. 6 is seen in experiments where a fiber is first subjected to an L-jump release to discharge tension-generating crossbridges. The sub-

sequent time-dependent return of the full amplitude of the phase-2 response was probed at various time intervals with small step releases (31). From our perspective, the interesting observation is that the phase-2 amplitude recovers with the kinetics of phase 3 (32). At the time, these data were interpreted in terms of a rapid detachment and reattachment of force-generating crossbridges (31, 32). We propose a simpler explanation in which the recovery of fiber tension results from the flux of new, non-tension-generating crossbridges (from step 1) through to the tension-generating transition—the overall process being governed by the kinetics of phase 3. Note that force is not generated with the kinetics of phase 3, which would be the case were tension generation coincident with phosphate release in step 1. Instead, phase-3 kinetics governs reestablishment of the equilibrium between non-force-generating and force-generating crossbridges in step 3 of our scheme. Changes in fiber stiffness are minor during the recovery process (31, 32), because the concentration of strongly bound non-force-generating and force-generating crossbridges, each contributing to fiber stiffness, changes little during tension recovery.

Relationship of the Mechanism to Water Oxygen–Phosphate Oxygen Exchange. Exchange experiments provide information on the strain dependence of the steps associated with phosphate release. In our system, strain is minimal because the various actomyosin states associated with phosphate binding and release are not subject to the wide range of strains typical of force-generating crossbridges. Our mechanism is consistent with the similar values for the ratio of the forward and reverse fluxes (R) that mediate intermediate (33) and medium (4) oxygen exchange in contracting muscle fibers and intermediate exchange with actomyosin in solution (34). These data are less easy to accommodate in mechanisms that associate tension generation with phosphate release. Here, the similar values of R can be accommodated only if the phosphate release step is made strain independent and various other mechanistic constraints are applied (4).

The Mechanism and the Maximum Velocity of Fiber Shortening. In contrast to the large reduction is isometric tension, the V_{max} or the unloaded shortening velocity of a muscle fiber is little changed by the addition of phosphate even at very high (52 mM) concentrations (2, 35). We observe a similar phosphate insensitivity for *de novo* tension generation by phase 2_{slow} and its equivalent, the medium speed T-jump relaxation ($1/\tau_2$). Likewise, Q_{10} values for *de novo* tension generation and for V_{max} are similar in value (36). An insensitivity of V_{max} to phosphate is expected for a mechanism like ours in which phosphate does not bind to crossbridge states that generate tension. Hitherto, the insensitivity of V_{max} to phosphate was thought to arise from a predominance of force-generating crossbridges at low strain with a reduced affinity for phosphate.

Conclusion. There is good evidence that the free energy of ATP hydrolysis is indirectly coupled to tension generation (37), which occurs as the result of an isomerization between crossbridge states with bound ADP. It is worth noting that rigor contraction, which resembles physiological contraction in a number of ways and takes place when muscle fibers are heated above their physiological temperature, also occurs in the absence of chemical change (14, 18). Although the scheme we present has a somewhat different role for product release, the plausible analogy between a clock escapement and muscle contraction considered by Huxley seems appropriate (38). Phosphate release (step 1) tensions the main spring, the irreversible isomerization (step 2) functions as the escapement, and tension generation (step 3) corresponds to the movement of the hands and the functioning of the clock.

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