A Single Order-Disorder Transition Generates Tension during the Huxley-Simmons Phase 2 in Muscle

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ABSTRACT Increasing temperature was used to progressively interconvert non-force-generating into force-generating states in skinned rabbit psoas muscle fibers contracting isometrically. Laser temperature-jump and length-jump experiments were used to characterize tension generation in the time domain of the Huxley-Simmons phase 2. In our experiments, phase 2 is subdivisible into two kinetic steps each with quite different physical properties. The fast kinetic component has rate constant of 950 s^{-1} at 1°C and a Q_{10} of ~1.2. Its rate is tension insensitive and its normalized amplitude declines with rising temperature—behavior that closely parallels the instantaneous stiffness of the cross-bridge. It is likely that this kinetic step is a manifestation of a damped elastic element/s in the fiber. The slow component of phase 2 is temperature-dependent with a Q_{10} of ~3.0. Its rate is sensitive to tension. Unlike the fast component, its amplitude remains in fixed proportion to isometric tension at different temperatures indicating direct participation in tension generation. Similar T-jump studies on frog fibers are also included. The combined results (frog and rabbit) suggest that tension generation occurs in a single endothermic (entropy driven) step in phase 2.

INTRODUCTION

Our interest in the use of changes in temperature to probe muscle contraction arose from the observation that rigor fibers of rabbit muscle, depleted of all ATP, contract when heated a few degrees above the working temperature of the muscle (Davis and Harrington, 1987a). At the time, we noted that the rate constants and amplitudes of tension generation in active fibers at low temperatures, and in rigor fibers above the working temperature of the muscle, were within factors of two to three of each other. Equivalent amounts of tension were developed in temperature-jumps of fibers undergoing either normal, or heat induced, contraction. Tension generation in rigor fibers is thus endothermic and arises from an order-disorder transition. A first step in establishing whether these findings are relevant to muscle contraction is to determine if force generation in activated fibers is also endothermic. Hitherto, the primary events of tension generation in activated fibers have been variously described as temperature-independent in rabbit fibers (Abbott and Steiger, 1977; Goldman et al. 1987) and temperature-dependent in frog (Ford et al., 1977; Gilbert and Ford, 1988) and rabbit fibers (Davis and Harrington, 1987a). In this paper, we resolve the controversy by showing that a single primary force generating step is endothermic in both rabbit and frog fibers.

Much of our current understanding of the primary events of tension generation has come from kinetic experiments in which a contracting muscle fiber is subjected to small, abrupt changes in length. Huxley and Simmons (1971) devised these experiments and showed that, when a fiber of frog muscle, contracting isometrically, is subjected to a step change in length there is an immediate change in tension called phase 1. This spring-like response was attributed to an elastic element resident in the cross-bridge. It is followed by phase 2 during which a significant proportion of the initial isometric tension, lost during phase 1, is recovered in a kinetically controlled reaction lasting a few milliseconds. Tension recovery during phase 2 is thought to consist of two to four kinetic steps that have been ascribed to a series of force generating structural changes in the attached cross-bridge (Ford et al., 1977). Various mechanisms of contraction, consistent with these mechanical data, have been proposed (e.g., Huxley and Simmons, 1971; Harrington, 1979; Huxley and Kress, 1985).

In the experiments described in this paper, fiber temperature was raised in order to progressively interconvert non-force-generating states to force-generating states. Resultant changes in the rates and amplitudes of key reactions of phases 1 and 2 were then probed in laser T-jump (temperature-jump) and L-jump (length-jump/step) experiments with the aim of extending our understanding of the physical nature of the primary events of force generation. States that generate tension or bear tension (either transiently or continuously) are all perturbed by changes in fiber length. Response to a T-jump requires the additional property of temperature sensitivity. Collectively, experiments of this type can provide useful information on the structures and structural transitions that generate tension. Purely elastic elements composed of random-coil polypeptide can be identified because they are readily perturbed by changes in length; minimally by temperature (see Harrington et al., 1990). On the other hand, structural changes in which an order-disorder transition dominates the energetics will respond to perturbation by both temperature and length. Endothermic reactions of this type

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can arise either from an increase in the conformational entropy of the polypeptide chain or when ordered solvent molecules (predominantly water) are disordered by displacement from the surface of the protein (as in protein assembly). As we shall see, the response of all but one of the identifiable steps of phases 1 and 2 appear to have properties that resemble an elastic element.

Since almost all the L-jump data has been collected on frog fibers it is necessary for our purposes to correlate the kinetic events that comprise phase 2 in the frog with their counterpart steps in rabbit fibers. In frog fibers it is generally accepted that the kinetically controlled tension recovery in the millisecond time domain constitutes phase 2. Evidence is presented to show that the expectation that phase 2 should likewise be fast in rabbit fibers led to the exclusion of a slower, principal kinetic component from the phase 2 of rabbit fibers by Abbott and Steiger (1977) and Goldman et al. (1987). This interpretation led them to conclude that phase 2 (i.e., tension generation) has a Q_{10} of $\sim$1; a result at variance with the Q_{10} of $\sim$1.85 obtained by Ford et al. (1977) for the overall process in the frog. In this paper we resolve the apparent paradox by showing that phase 2 is comprised of reactions that have quite different temperature coefficients and therefore rates at different temperatures. Furthermore we show that the kinetics of frog and rabbit fibers can be correlated once it is appreciated that the rate of the temperature-dependent kinetic component of phase 2 scales, not with absolute temperature, but with the working temperature of the muscle (frog $\sim$25°C; rabbit 39.3°C). This means that at the same temperature, the temperature-dependent reaction of phase 2 will be slower in the rabbit than in the frog. Implicit in this observation is that tension recovery during phase 2 does not appear to be composed of a series of related reactions as once thought (Ford et al., 1977), but can be resolved into a single temperature-dependent and other temperature-independent rate processes.

The primary events of tension generation are fast and require special techniques for their study. Perturbation methods that rapidly alter either the temperature or length of single muscle fibers are therefore used in these experiments. Relaxation kinetic theory developed for the analysis of reactions perturbed minimally from a steady state is used to analyze the data (Bernasconi, 1976). Under such conditions, the L- and T-jump tension transients have the form of a sum of a series of exponentials. A laser T-jump, based on an iodine photodissociation laser, is used to identify and characterize temperature-sensitive reactions (Davis and Harrington, 1987b). The heating time is more than two orders of magnitude faster than comparable instruments (Goldman et al., 1987; Bershitsky and Tsaturyan 1989) and is particularly stable (within 2% of the T-jump over the 400 ms). The thermal gradient across the observation cell is small (6% of the T-jump). Abrupt changes (160 μs) in length are used to perturb fibers contracting isometrically. This rate of length change is typical of that used by other workers (e.g., Ford et al., 1977). Five relaxations result from a change in length; only two dominate the much simpler kinetic response to a T-jump. It should be noted that the laser T-jump leaves the geometry of the myosin and actin filaments unperturbed. A preliminary report on aspects of these experiments has been published (Davis and Harrington, 1991).

MATERIALS AND METHODS

Fiber preparation and solutions

Rabbits were first anesthetized with ketamine (Ketaset, Aveco Co., Inc., Fort Dodge, IA) at a dose of 50 mg/kg and then sacrificed. Glycerinated fibers were prepared from psoas muscle using the following procedure. Bundles of fibers approximately 1 mm in diameter were separated from the lateral edge of the psoas, tied to sticks at their in vivo length, before being cut free and placed in ice-cold skinning solution that contained 150 mM potassium propionate, 5 mM KH_2PO_4, 5 mM magnesium acetate, 3 mM vanadium free ATP, 5 mM EGTA, 1 mM dithiothreitol, and 0.2 mM NaN_3 adjusted to pH 7.0 at room temperature with 1.0 N KOH. Four sticks were placed in each 100-ml beaker with a magnetic stirrer and surrounded by ice. The skinning solution was changed four times at 2-h intervals after which the individual fiber bundles were transferred to capped tubes containing 50% glycerol and 50% double strength skinning solution and left on ice overnight before cooling to −20°C for longer term storage. Fibers were used for experiments within 10 days of preparation because the sarcomere diffraction pattern of fibers stored for longer periods appeared less stable at elevated temperatures. At the time of the experiment a short subsegment of the fiber bundle attached to the stick support was dissected free and placed in a dissecting dish containing Dow Corning 200 silicone oil, viscosity 10 cs (W. F. Nye, Inc., New Bedford, MA) at 5°C. Single fibers were dissected free using a combination of forceps and electrolytically sharpened tungsten needles.

Frog fibers were obtained from cold adapted *Rana temporaria*. Single fibers were dissected from the semitendinosus muscle under paraffin oil and were mechanically skinned using forceps and electrolytically sharpened tungsten needles. Thinner fibers were selected in order to enhance the rate of diffusion of the ATPase substrate and products in and out of the fibers.

Aluminum T-clips (Goldman and Simmons, 1984) were fitted $\sim$ 4 mm apart and were used to attach the fiber to the stainless steel mounting hooks in the tension transducer cell. Fiber lengths and diameters (at three positions along the fiber length) were measured in the transducer cell containing relaxing solution after setting the sarcomere spacing using the first order diffraction pattern generated by a 2 mW He/Ne laser (Spectra Physics, Mountain View, CA). Fiber activation was achieved by first washing with preactivating solution and then rapidly flowing activating solution into and through the cell at a temperature of 1°C (and on occasion 6°C). Excess solution was removed by suction at the opposite end of the trough of the laser T-jump and L-jump cell. Reference T-jump and L-step tension transients were first recorded at the low temperature before adjusting to the higher temperature of the experiment. Rabbit fiber tension measured at 6°C was 113 SD $\pm$ 44 kNm^{-2}, n = 10.

The solutions used differ in composition from those used in our earlier work insofar as glycerol 2-phosphate (Goldman et al., 1987) replaced borate as the temperature-insensitive buffer component. The ionic strength changed from 0.17 M in the earlier buffer to 0.2 M in the new. Relaxing solution: 7.52 mM $MgCl_2$, 5.48 mM vanadium free ATP, 20 mM EGTA, 20 mM creatine phosphate, 15 mM disodium glycerol 2-phosphate. Preactivating solution: 7.52 mM $MgCl_2$, 5.48 mM vanadium free ATP, 0.1 mM EGTA, 19.9 mM HDTA (1,6-diaminohexane-*N,N,N',N'*-tetraacetic acid), 19.9 mM creatine phosphate, 15 mM disodium glycerol 2-phosphate. Activating solution: 7.39 mM $MgCl_2$, 5.52 mM vanadium-free ATP, 20 mM CaEGTA, 20 mM creatine phosphate, 15 mM disodium glycerol 2-phosphate. All solutions contained 2 mg ml^{-1} of creatine phosphokinase, and were adjusted to pH 7.1 at room temperature with 1 N KOH (Goldman et al., 1987).

Tension measurements

Particular care was taken to minimize the development of structural disorder in the fibers. Fibers were not length-clamped because of the low signal to noise ratio that results when the requisite control circuitry is used during small length-step experiments, a situation further exacerbated by elevated temperatures. Ford et al. (1977) point out that the benefits of length-clamping are most evident when large step-changes in length are applied to fibers. We therefore chose, as they did, to perform small amplitude L-jump experiments under motor control alone. Goldman et al. (1987) used a similar protocol in their studies on the temperature dependence of the L- and T-jump kinetics of rabbit fibers. The experimental procedure we finally adopted was primarily designed to maintain reproducibility of the kinetics (rates and amplitudes) of the tension transients during the course of the experiment and to minimize obvious (to the eye) time-dependent changes in the sarcomere diffraction pattern. A dissecting microscope, positioned over the fiber, was also used to monitor fiber quality. Eight L-jump and four T-jump tension transients were recorded sequentially at a selected temperature, after which the fiber was discarded. The individual tension transients were inspected before averaging in order to check for the time-dependent deterioration of the fibers. Data were discarded if nonrandom changes were detected in the kinetic parameters. Perturbation experiments were carried out on four or more individual fibers at each temperature. Each fiber was exposed to the elevated temperature for less than 3 min. The self-consistency of the kinetic parameters recorded in T- and L-jump experiments at lower temperatures where activated fibers were stable for extended periods of time and at high temperatures where fibers were less stable also helped to validate the technique.

Data obtained from control experiments in which the fiber ends were cross-linked with glutaraldehyde (Chase and Kushmerick, 1988) to reduce fiber end-compliance and the sarcomere pattern was stabilized using the procedure of Brenner (Brenner, 1983; Sweeney et al., 1987) were used for comparison purposes. Experimental details are provided in the legend to Fig. 5. In these experiments, changes in sarcomere length and tension were simultaneously recorded during the course of the reaction. Kinetic data from these well preserved fibers with low end-compliance and stable sarcomere patterns gave rates and amplitudes virtually indistinguishable from data collected using the less elaborate protocol described earlier in this section thereby validating the simpler method used in the paper to obtain the kinetic data

The fiber cuvette used for both the T-jump and L-jump measurements was similar in design to that described by Davis and Harrington (1987b). It was modified for the experiments described by replacing the stainless steel fin (*T* in Fig. 1 of Davis and Harrington, 1987b) used previously with a copper fin fitted with a 2 × 2 × 15-mm stainless steel cap. Stainless steel alone contacts the medium that bathes the fiber. Rapid heating and cooling of the fiber was achieved by using a Peltier (thermoelectric) temperature control. The above modifications allowed the temperature of the fiber to be raised rapidly, taking an average of 55 s to go from 1° to 40° ± 0.25°C; heating times from the starting temperature of 1°C were linearly proportional to the final temperature. The diffraction pattern obtained from a He/Ne laser was used to set and monitor sarcomere spacing.

A Model 407A (Cambridge Technology Inc., Cambridge, MA) capacitor-based force transducer with a resonant frequency of 12 KHz, 100 μs rise time and compliance of 0.1 μm g^{-1} was used for all tension measurements. The thermal stability of the transducer was significantly improved by using a thermoelectric cooling device (Power supply P/N 809-3020-01-00-00 and Camcool thermoelectric module P/N 852-1547-04; Interconnection Products, Inc., Santa Ana, CA) to maintain the temperature of the unit at 20 ± 0.1°C.

Laser T-jump

The fiber in the 2 × 2 × 15-mm cuvette is heated uniformly by the 1.315-μm infrared beam in less than 1 μs. The laser T-jump heated the fiber 5°C with a 1.7-J pulse of infrared light. The optics were set such that the temperature of the T-jump cell remained within 6% amplitude of the T-jump (5°C) for 800 ms. Post-jump temperature was monitored at different positions normally occupied by the fiber with a 0.001 in copper constantan fine wire thermocouple (Omega Engineering Inc., Stamford, CT). Details of the design and construction of the infrared laser and its associated optics are described fully elsewhere (Davis and Harrington, 1987b).

L-jump

An ergometer (Model 300S; Cambridge Technology, Inc., Cambridge, MA) is used for the L-jump experiments. In the −1.5 μm per half sarcomere L-jump experiments used in this paper the 100% rise time of the step motor is 160 μs. The instrument was modified so that the step response could be fine-tuned externally to the instrument. A programmable function generator (Model 3314A; Hewlett-Packard, Marysville, WA) was used to drive the ergometer and to trigger the start of data collection. A series of eight step stretches and releases were recorded; 5 s was allowed to elapse for tension recovery before the next L-jump was applied to the fiber.

Data collection and analysis

The voltage output from the force transducer amplifier is recorded on a digital oscilloscope (Model 430; Nicolet Instrument Corporation, Madison, WI). Variable length records (generally 8000 data points) of 12-bit data were recorded. These data are transferred to a Hewlett-Packard 1000 computer for storage and analysis. Experimental records were analyzed using a non-linear least squares routine (Johnson and Frazier, 1985). In order to do this, 900- or 450-point records, spaced at logarithmic intervals, were generated from the full length records using the Savitzky-Golay smoothing routine to average the data adjacent to the selected time intervals (Savitzky and Golay, 1964). Tension relaxations were usually analyzed in their entirety.

RESULTS

Analysis of laser T-jump and L-jump relaxation spectra

Laser T-jump experiments were performed in order to detect and to obtain the kinetic parameters (rates and amplitudes) of the temperature-sensitive reactions that lead to tension generation. These results are contrasted with L-jump kinetics in which *all* states that can or do bear tension are subjected to mechanical perturbation. Experimentally this is achieved by performing T-jumps and L-jumps at a series of temperatures so that "kinetic signatures" of the temperature dependence of various kinetic steps can be obtained (for examples see Figs. 7–9). This allows individual T-jump and L-jump kinetic steps to be cross-correlated so that the physical nature of the various transitions can be determined from an analysis of their specific responses to length and temperature perturbation. We have also included a control experiment (see Fig. 5) to show that kinetic data from fibers with reduced end-compliance and stable sarcomere patterns give rates and amplitudes virtually indistinguishable from data collected with the less elaborate fiber handling protocol used to obtain the bulk of the T-jump and L-jump kinetic data in this paper.

Different symbols are used for the reciprocal relaxation times or apparent rate constants obtained from the T-jump and L-jump experiments; for example, $1/\tau_1$ refers to the fastest relaxation seen in T-jump experiments; k'_1 to the equivalent relaxation seen in L-jump experiments. These assignments were, of course, made after completing and interpreting the experiments described in the paper. Our

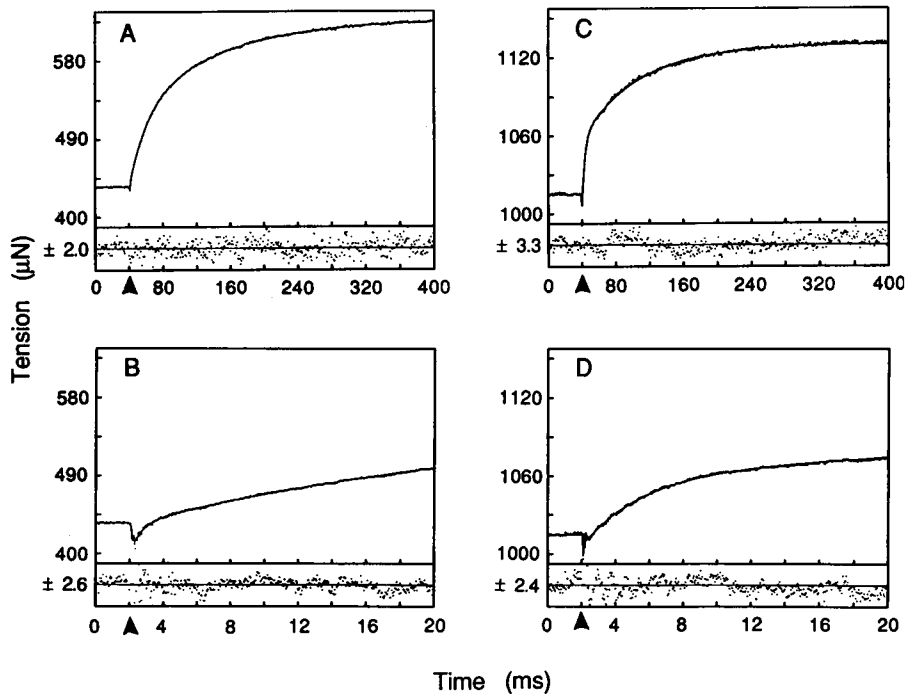

FIGURE 1 Kinetics of tension generation following a laser T-jump of a rabbit psoas fiber undergoing isometric contraction at two different temperatures. The response to a T-jump of 5°C at post-jump temperatures of 6°C (*A* and *B*) and 21°C (*C* and *D*) are illustrated at slow (*top*) and fast (*bottom*) time bases. Two consecutive T-jump relaxations were averaged in each case. Note the step drop in tension followed by a fast recovery of tension ($1/\tau_1$) at 6°C (*B*); at 21°C (*D*) $1/\tau_1$ appears absent. The experimental data are plotted in point form, the continuous fitted line drawn through these data was computed from: tension drop = −33 μN, $1/\tau_1$ = 1549 s^{-1}, a_1 = 32 μN, $1/\tau_2$ = 46.5 s^{-1}, a_2 = 83 μN, $1/\tau_3$ = 9.6 s^{-1}, a_3 = 106 μN for *A* and *B* and $1/\tau_2$ = 289 s^{-1}, a_2 = 51 μN, $1/\tau_3$ = 14.3 s^{-1}, a_3 = 72 μN for *C* and *D*. The residuals plotted below each transient were obtained by subtracting the fitted line from the experimental data. The arrow indicates the point at which the T-jump was applied. Sarcomere spacing was 2.6 μm.

prime concern is with the fast reactions of phases 1 and 2 seen in Huxley-Simmons length-step experiments: slower kinetic events are, however, also included in the analysis for technical reasons and will form the subject of a subsequent publication.

T-jump kinetics of rabbit and frog fibers

Literature reports describe T-jump tension transients of activated fibers as biexponential (Goldman et al., 1987; Davis and Harrington, 1987a; Bershitsky and Tsaturyan, 1988, 1989, 1992). This can be confusing because three exponentials are often resolved (Davis and Harrington, 1991). In order to clarify these issues, the four phase tension transients seen in our T-jump experiments on rabbit and frog fibers are described first. The relationship of these phases to other reports in the literature is reserved for the Discussion.

Responses of single, skinned rabbit psoas and frog semitendinosus fibers contracting isometrically to a T-jump are illustrated in Figs. 1–3, respectively. Each reaction profile for the rabbit is the average of two successive T-jumps of 5°C on one fiber; the frog data were obtained from a single T-jump of 2.5°C. The rabbit and frog data can be compared, because the perturbation from equilibrium by the T-jump is small in both instances; conditions under which the size of the T-jump has little effect on the rates of the kinetics. Four distinct phases can be resolved at low and moderate temperatures. Kinetic parameters are always specified at the post-jump temperature, because the tension transients occur after the T-jump. Early events are best seen in records at low temperatures using a fast time-base (Figs. 1, *B* and *D*; 2 *B*; 3 *B* and *D*). The tension transient begins with a virtually instantaneous drop in tension that tracks the response time of the force transducer. A low amplitude, fast recovery of tension ($1/\tau_1$) is frequently present at low temperatures in both rabbit and frog fibers. This response is clearly evident in the fast time-base, low temperature records of Figs. 1 *B*; 2 *B*; and 3 *B*. It can also be seen in the frog T-jump records obtained at the higher temperature illustrated in Fig. 3 *D*, but was not fitted during data analysis, because the tension transients

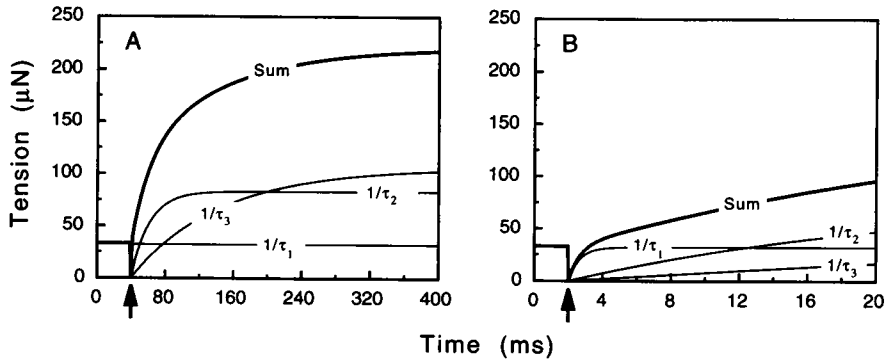

FIGURE 2 Simulation of the three kinetic phases observed after a laser T-jump of a rabbit psoas fiber undergoing isometric contraction. These data are illustrated at slow (*A*) and fast time-bases (*B*) so that the relative contributions of the different components of the reaction can be readily judged. The reaction profiles were simulated using the kinetic and amplitude data from the T-jump tension transients of Fig. 1, *A* and *B*, at a post-jump temperature of 6°C (tension drop = −33 μN, $1/\tau_1$ = 1549 s^{-1}, a_1 = 32 μN, $1/\tau_2$ = 46.5 s^{-1}, a_2 = 83 μN, $1/\tau_3$ = 9.6 s^{-1}, a_3 = 106 μN).

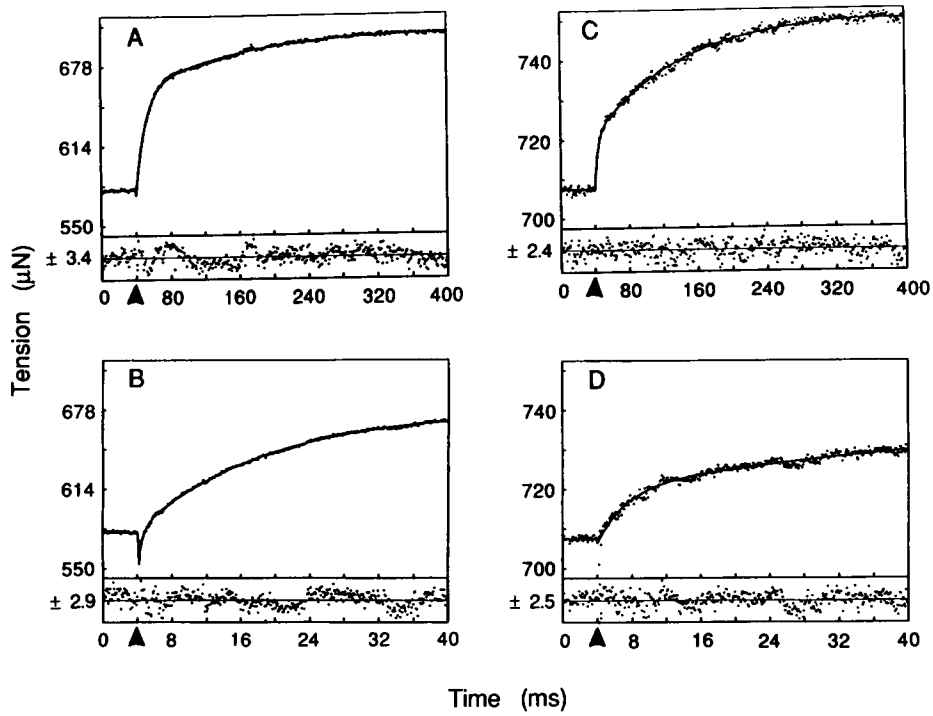

FIGURE 3 Kinetics of tension generation following a laser T-jump of a frog semitendinosus fiber undergoing isometric contraction at two different temperatures. The response to a T-jump of 2.5°C at post-jump temperatures of 3.5°C (*A* and *B*) and 13.5°C (*C* and *D*) are illustrated at slow (*top*) and fast (*bottom*) time bases. Note the step drop in tension followed by a fast recovery of tension ($1/\tau_1$) at 3.5°C (*B*); at 13.5°C (*D*) the fast recovery is present but is not fitted to the data for technical reasons. The experimental data are plotted in point form, the continuous fitted line drawn through these data was computed from: tension drop = −29 μN, $1/\tau_1$ = 1840 s^{-1}, a_1 = 31 μN, $1/\tau_2$ = 94 s^{-1}, a_2 = 82 μN, $1/\tau_3$ = 8.5 s^{-1}, a_3 = 49 μN for A and B and $1/\tau_1$ and a_1 not determined, $1/\tau_2$ = 302 s^{-1}, a_2 = 14 μN, $1/\tau_3$ = 9.7 s^{-1}, a_3 = 31 μN for *C* and *D*. The residuals plotted below each transient were obtained by subtracting the fitted line from the experimental data. The arrow indicates the point of the T-jump. Sarcomere spacing was 2.2 μm.

were recorded with older equipment and consisted of too few data points. Fast recovery appears absent in the rabbit T-jump data recorded at 21°C illustrated in Fig. 1 *D*. It is worth noting that these low amplitude kinetic events are relatively minor components compared to the large amplitude biexponential relaxation that follows and dominates the slow time-base records of Figs. 1, *A* and *C*; 2 *A*; 3 *A* and *C*. The amplitudes of both these relaxations decline with rising temperature. Of the two relaxations, the faster is termed the medium speed relaxation ($1/\tau_2$), the other the slow speed relaxation ($1/\tau_3$).

Kinetic parameters were usually obtained by fitting these data to three exponentials. In instances where the fast relaxation was difficult to detect, the kinetics of the biexponential phase was determined by fitting the tension versus time records after 2 ms had elapsed. Contributions from the low amplitude 1000 to 2000 s^{-1} relaxation are insignificant under these conditions. When present, the amplitude associated with $1/\tau_1$ is consistently lower than the amplitude of the step drop in tension that precedes it (see Figs. 1–3). The decline in the amplitude of the fast relaxation ($1/\tau_1$) below the limits of detection in the T-jump experiments at higher temperatures correlates with results obtained from L-jump experiments in which the relative amplitude of k'_1 (the L-step equivalent of $1/\tau_1$) decreases similarly with temperature (see Fig. 7). Inspection of the residuals plotted below each tension transient in Figs. 1 and 3, shows that the computed kinetic parameters fit the data well. Our concern is with the fast ($1/\tau_1$) and medium speed ($1/\tau_2$) relaxations which we intend to show are, respectively, the temperature-insensitive, and temperature-sensitive, components of phase 2.

Length-jump relaxation kinetics of rabbit fibers

L-jump experiments were performed in order to characterize reactions sensitive to cross-bridge states that either bear, or transiently bear, tension. A small L-step of −1.5 nm per half sarcomere was selected, because it perturbed the system minimally. This allows the kinetics to be analyzed as a series of coupled linear (first-order) differential equations. Experiments are carried out at different temperatures so that the kinetic steps seen in L-jump experiments and in the earlier described T-jump data can be correlated. In frogs, the kinetic response to a L-jump is complex in form and has been subdivided into phases 1, 2, 3, and 4 (Ford et al., 1977). Abbott and Steiger (1977) and Goldman et al. (1987) concluded that, in the rabbit, unlike the frog, the kinetics of phase 2 were only marginally sensitive to temperature. Later in the paper, we make the point that these conclusions are probably incorrect and result from excluding a temperature-dependent kinetic step from phase 2.

Our approach to the analysis of these data is different from that employed by others in that we have chosen to fit the overall kinetics of phases 2, 3, and 4 to the sum of four exponentials. The advantage is that the contribution of each kinetic component to the overall amplitude of the reaction at all times is well defined. This is particularly important when comparing reaction steps whose rates change independently of each other with temperature and improves on frequently used methods in which half times are estimated for complex processes and reaction amplitudes are determined by the linear back extrapolation of subsequent slower phases to zero time. Since the L-jump applied by the ergometer has the form of a linear ramp, time zero for the start of the reaction is taken as the midpoint of the transient drop in tension during phase 1.

Time courses of tension recovery at 6 and 21°C in rabbit fibers are illustrated in Fig. 4, *A*, *B*, *C*, and *D*, respectively. Eight L-jump records were computer-averaged to produce each transient. The quality of the four exponential fit to the L-jump data can be judged from the random distribution of

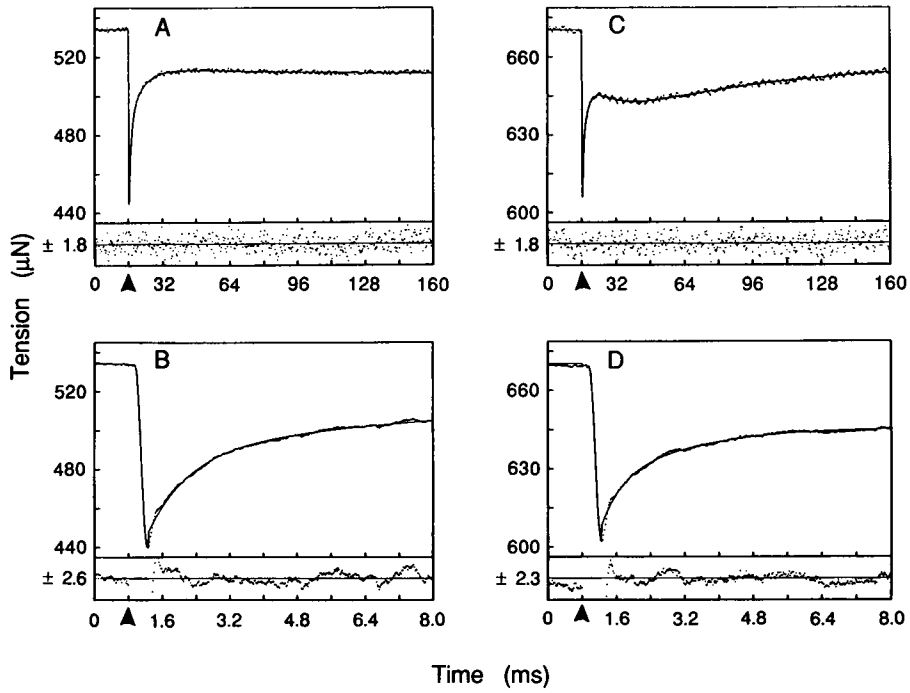

FIGURE 4 Kinetics of tension generation following the L-jump of a rabbit psoas fiber undergoing isometric contraction at two different temperatures. The response of a fiber at 6°C at a slow (*A*) fast time bases (*B*) and at 21°C at a slow (*C*) and fast time base (*D*) to a L-jump of −1.5 nm per half sarcomere in 160 μs is illustrated. Eight relaxations were averaged in each case. The arrow indicates the point of the L-jump; residuals plotted below each transient were obtained by subtracting the fit to H-S phases 2, 3, and 4 from the experimental data. Sarcomere spacing was 2.6 μm.

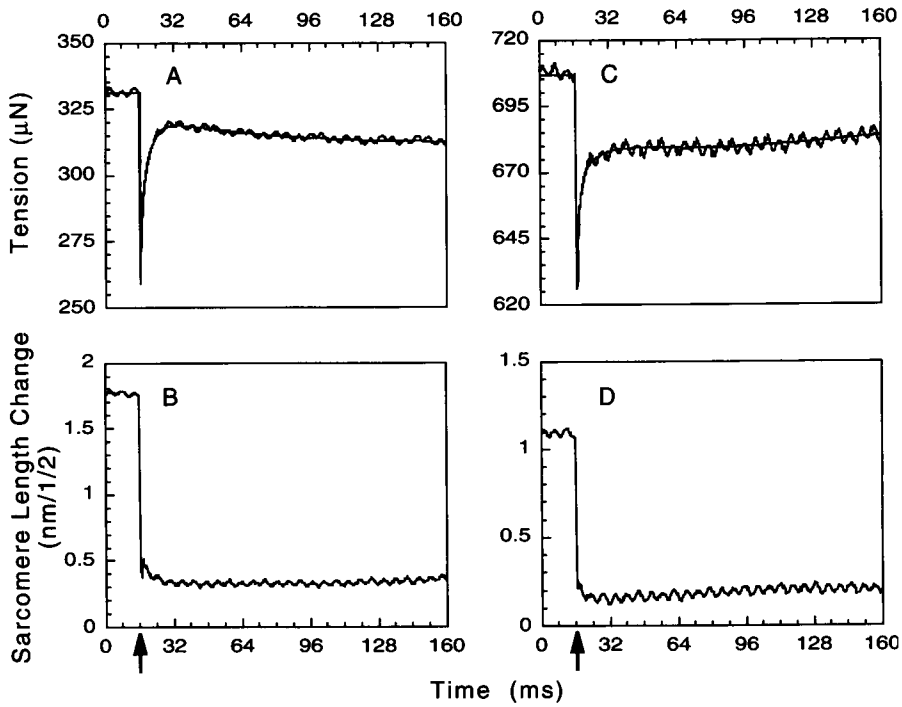

FIGURE 5 Kinetics of tension generation and the associated changes in sarcomere length following the L-jump of a rabbit psoas fiber undergoing isometric contraction at two different temperatures. The fiber ends attached to the clips were treated with glutaraldehyde (Chase and Kushmerick, 1988) to reduce end-compliance and the length cycling procedure of Brenner (Brenner, 1983; Sweeney et al., 1987) was applied to reduce sarcomere length dispersion during activation. Changes in sarcomere length were recorded with a 10 mW He/Ne laser set at the Bragg angle to the fiber and a SL15 super linear position sensor and Model 301DIV-30 position sensing amplifier (UDT Sensors, Hawthorne, CA) to follow changes in the diffraction pattern. Note the stability of the length of the sarcomere following the applied length-jump. The response of a fiber at 1°C (A and B) with $k_1' = 1512\ s^{-1}$, $a_1 = 35.1\ \mu N$, $k_2' = 246\ s^{-1}$, $a_2 = 43.7\ \mu N$, and normalized T_1 and T_2 values of 0.73 and 0.97, respectively and at 11°C (C and D) with $k_1' = 844\ s^{-1}$, $a_1 = 52.4\ \mu N$, $k_2' = 102\ s^{-1}$, $a_2 = 18.9\ \mu N$, and normalized T_1 and T_2 values of 0.87 and 0.97, respectively. As can be seen the kinetic constants and amplitudes obtained from the analysis of these control experiments differ little from the data presented in the body of the paper employing a less rigorous protocol, thereby validating the procedure employed. The arrow indicates the point of the L-jump.

the residuals about the mean that are plotted beneath each tension transient. Fig. 5 shows control L-jump experiments at 6° and 11°C in which the sarcomere stability was enhanced and end-compliance was reduced (see details in legend). As can be seen, there is little change in the sarcomere length during the acquisition of the tension record. Comparison of the kinetic data of Fig. 5 with those of Fig. 4 and related experiments at various temperatures show that the kinetic and amplitude parameters obtained by the two methods are virtually indistinguishable. Huxley-Simmons T_1 values are obtained by back extrapolation of all resolvable kinetic phases to zero time. These values are only marginally lower than those determined by direct measurement of the extremity of phase 1. The reason for this is that there is a degree of overshoot in the values for the extremity of T_1 obtained directly from the experimental records (see residuals of Figs. 4, *B* and *D*). Early stages of tension recovery are dominated by the fast exponential component of phase 2 (k'_1) which is seen to best advantage in the fast time base records in the Fig. 4, *B* and *D*. The step correlates with the small amplitude fast relaxation ($1/\tau_1$) seen in the laser T-jump experiments. The residuals do show some ringing of the force signal in the first 500 μs, but this decays rapidly and interferes minimally with analysis. The well resolved slow exponential component of phase 2 (k'_2 of 80 to 700 s^{-1}) follows. We later present evidence to show that this step correlates with the large amplitude medium speed relaxation ($1/\tau_2$) seen in the laser T-jump. The computed endpoint of the reactions comprising phase 2 provide values for the Huxley-Simmons T_2 at different temperatures. Slower events of phases 3 and 4 were fitted to the data so that their contributions could be separated from the kinetic events of phases 1 and 2. Kinetic coupling between phase 2 and phases 3 and 4 is minimal, since the latter reactions equilibrate to the new fiber length at an order of magnitude slower rate.

Temperature dependence of Huxley-Simmons T_1

The temperature dependence of fiber elasticity is manifest in the T_1 values illustrated in Fig. 6. Typical L-jump data used for these measurements are shown in Fig. 4. It is evident from the graph that fiber stiffness does not rise in concert with tension as the temperature of the fiber is raised. These data are important because they serve as an example of the temperature dependence of an elastic element located predominantly in the cross-bridge (Ford et al., 1981). T_1 values were obtained as described in the previous section. It is unlikely that the observed decline in the normalized values for T_1 (i.e., $1 - T_1$) would arise from the truncation of the fall in tension by rapid recovery during phase 2. This is because, as we shall see in the next section, the rate of k'_1, the fast L-jump component of phase 2 increases less than a factor of two (1000–1700 s^{-1}) over the 30°C temperature range. The temperature dependence of T_2, also illustrated in Fig. 6, is discussed later.

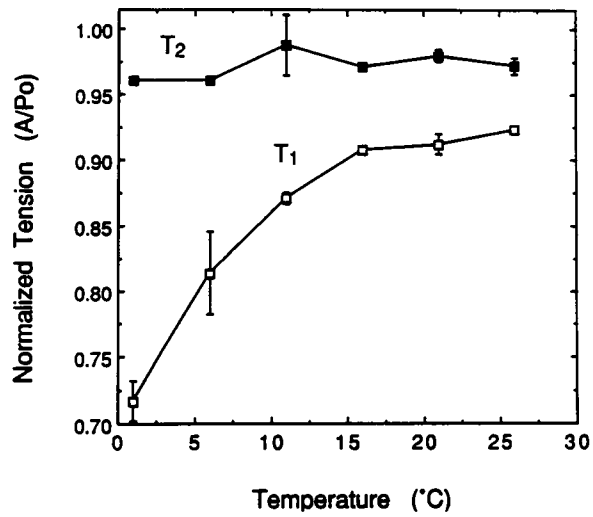

FIGURE 6 Temperature dependence of T_1 the tension minimum attained during phase 1, and T_2 the tension maximum reached after phase 2 recovery in rabbit psoas fibers. These data were obtained from experiments similar to those depicted in Fig. 4. Tensions are normalized to isometric tension (P_0) at the temperature of the experiment. Each error bar (hidden within the symbols on occasion) represents the standard deviation of the T_1 and T_2 values obtained from experiments on a minimum of four fibers.

Temperature dependence of the L- and T-jump kinetics of the fast component of phase 2

Plots of the relaxation rate constants and reaction amplitudes versus temperature after a 160-μs L-jump of −1.5 nm per half-sarcomere are illustrated in Fig. 7. The k'_1 values obtained are virtually independent of whether a 1.5-nm per half-sarcomere stretch (data not shown) or a −1.5 nm per half-sarcomere release is applied to the fiber. Inspection of Fig. 7 shows that the temperature dependence of k'_1 is not Arrhenius in form. This is to be expected since the relaxation rate constant k'_1 in a small perturbation relaxation experiment is comprised of the sum of the forward and reverse rate constants that govern the overall reaction. Apparent Arrhenius behavior is more the exception than the rule under such circumstances. Q_{10} values are likewise approximate, but are included since they are frequently used to characterize muscle kinetics in physiological experiments. A Q_{10} of ~1.2 holds at lower temperatures, reflecting the close to temperature-independent nature of the relaxation seen by us and others (Abbott and Steiger, 1977; Goldman et al., 1987).

T-jump experiments (Figs. 1–3) confirm the temperature-independent nature of the reaction associated with k'_1 in rabbit fibers. The only relaxation detected in the time domain of k'_1 is $1/\tau_1$ which appears, as discussed earlier, to arise as the result of a tension drop due to fiber expansion and not from the direct effect of temperature on the cross-bridge cycle. Frog fibers (Fig. 3) exhibit a similar kinetic response to a T-jump in the same time domain. The similar physical origin for k'_1 and $1/\tau_1$ was first commented on by Goldman et al. (1987) in experiments on rabbit fibers.

Inspection of Fig. 7, reveals that the normalized amplitude of the reaction associated with k'_1 declines with rising temperature. The temperature dependence parallels changes in

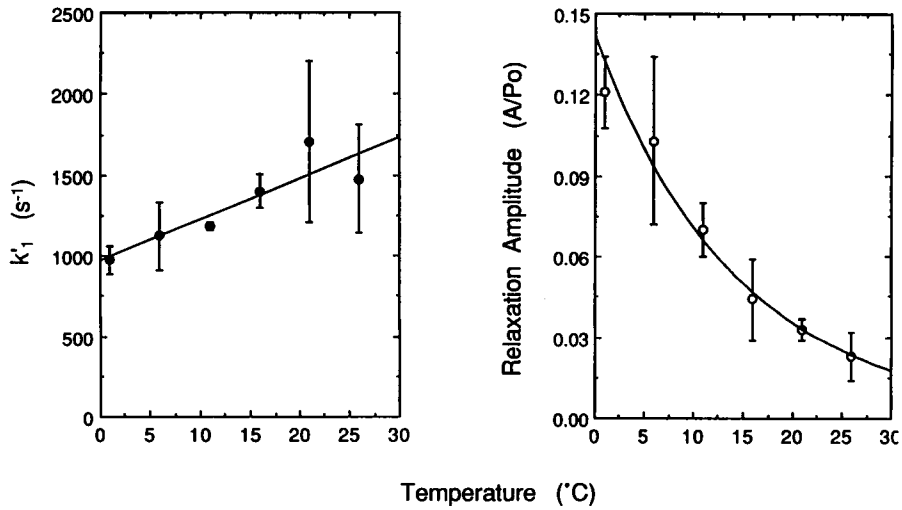

FIGURE 7 Temperature dependence of the L-jump kinetics of the fast kinetic component of the H-S phase 2. These data were obtained from experiments that include those depicted in Fig. 4. Reaction amplitudes are normalized to the isometric tension (P_0) at the temperature of the experiment. Each error bar represents the standard deviation of rate or amplitude data obtained from experiments on a minimum of four fibers.

the T_1 (i.e., $1 - T_1$) response illustrated in Fig. 6. The correlation of the reaction amplitudes associated with k'_1 and fiber stiffness (the T_1 response) suggests that the fast component of phase 2 has more the physical properties of a damped elastic element than of a force generating transition in the crossbridge.

Temperature dependence of the L- and T-jump kinetics of the slow component of phase 2

In this section we establish that k'_2 seen in L-jump experiments and $1/\tau_2$ seen in T-jump experiments are manifestations of the same process. Fig. 8 illustrates the temperature dependence of the rate and amplitude of the slow exponential component of Huxley-Simmons phase 2. The temperature dependence of k'_2 exhibits several distinctive features: Between 1° and 11°C in these L-jump experiments, k'_2 declines as temperature rises; above 11°C k'_2 increases in a linear manner with temperature with an approximate Q_{10} of 3.0. This is distinctly non-Arrhenius behavior. The atypical negative temperature dependence of k'_2 at low temperatures is indicative of coupling to an adjacent reaction (Bernasconi, 1976) in the cross-bridge cycle. The discontinuity is mirrored in the temperature dependence of the reaction amplitudes depicted in the right-hand panel of Fig. 8. A point worthy of note, is that the amplitude of the step appears to be a constant fraction of the isometric tension above 6°C. That is, the amplitude of the reaction is directly proportional to isometric tension once the step is uncoupled from an interfering adjacent step. Contrast this with the quite different decline ($1 - T_1$) in amplitude of the T_1 values of Fig. 6 and the amplitude of k'_1 of Fig. 7. It should be noted that k'_1 and k'_2 become equivalent in rate and are difficult to resolve experimentally at temperatures much above 30°C in the rabbit.

The temperature dependence of the rates and amplitudes of $1/\tau_2$ obtained from laser T-jump experiments is illustrated in Fig. 9. Comparison of the temperature dependence of $1/\tau_2$ with that of k'_2 in the left-hand panels of Figs. 8 and 9, respectively, reveals similarities. A break in the kinetics at 11°C, though less marked in the T-jump data, is a common feature. An almost linear dependence of the relaxation rate constants at temperatures at and above 11°C is evident in

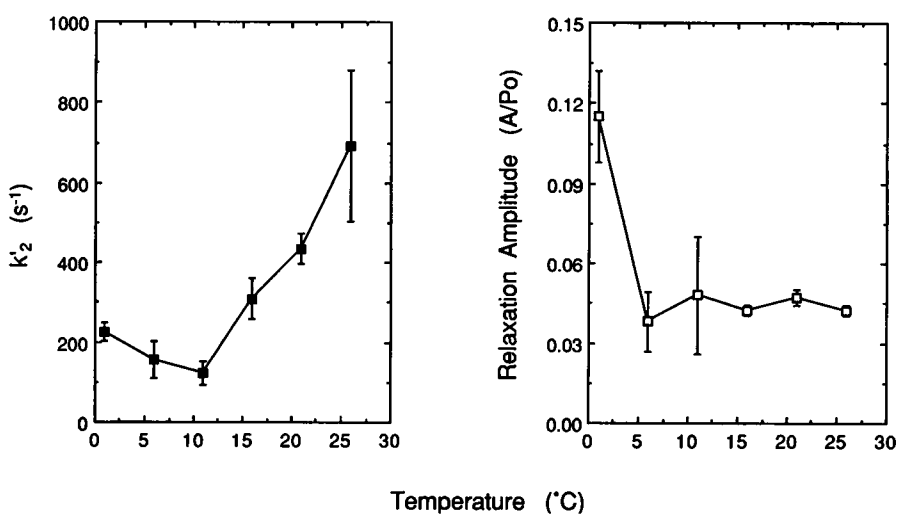

FIGURE 8 Temperature dependence of the L-jump kinetics of the slow kinetic component of the H-S phase 2. Note the similar form of the temperature dependence of the relaxation rate constant, k'_2 to that of $1/\tau_2$ in the T-jump experiments of Fig. 9. These data were obtained from experiments that include those depicted in Fig. 4. Reaction amplitudes are normalized to the isometric tension (P_0) at the temperature of the experiment. Each error bar represents the standard deviation of rate or amplitude data obtained from experiments on a minimum of four fibers.

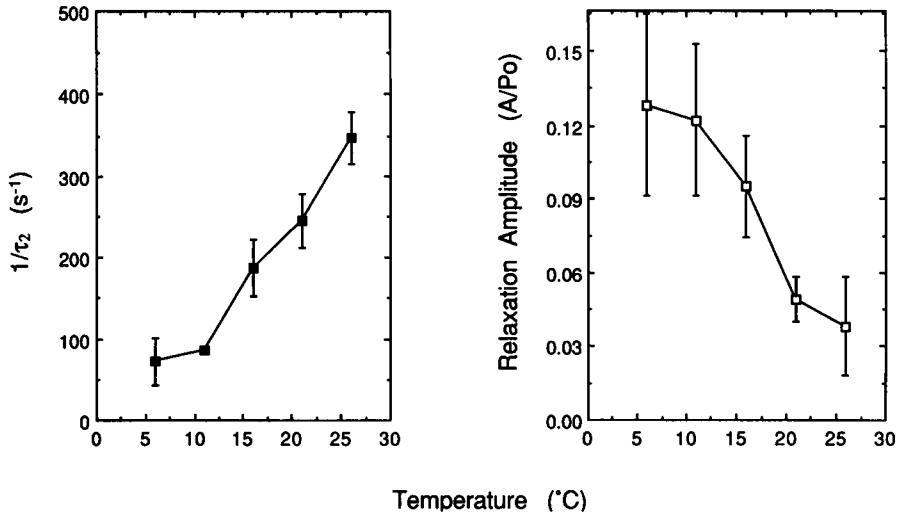

FIGURE 9 Temperature dependence of the T-jump kinetics of the slow kinetic component of the H-S phase 2. Note the similar form of the temperature dependence of the relaxation rate constant, $1/\tau_2$ to that of k_2' in the L-jump experiments of Fig. 8. These data were obtained from experiments that include those depicted in Fig. 1. The kinetic parameters are plotted at the post-jump temperature. Reaction amplitudes are normalized to isometric tension (P_0) at the pre-jump temperature and the error bars represent the standard deviation of the rate and amplitude data obtained from experiments on a minimum of four fibers.

both data sets. Rates are a factor of two lower in the T-jump experiments. This is probably due to the fact that kinetics of phase 2 of L-jump experiments typically increase in rate the greater the step-release; experiments in which the fiber is stretched and tension rises exhibit a slowing of rate proportional to the extent of stretch. It is interesting to note that k_2' and not k_1' (see Fig. 7) exhibits a marked tension sensitivity of rate. This is reflected in the observation made earlier that the values of k_1' are virtually independent of whether a 1.5 nm per half sarcomere stretch or release is imposed on the fiber. T-jump relaxation amplitudes normalized to pre-jump isometric tension (Fig. 9) have a different form to the normalized L-jump amplitudes of Fig. 8. This is because the reaction amplitudes arising from a T-jump are normalized to a fixed isometric tension recorded at the pre-jump temperature while the L-jump amplitudes are recorded at and normalized to the isometric tension at the same temperature. The decline in the T-jump amplitudes of Fig. 9 results from a decrease in reaction amplitude generated by a 5°C change in temperature relative to the temperature-induced increase in isometric tension.

Evidence that k_2' and $1/\tau_2$ are kinetic components of the H-S phase 2 in rabbit and frog

In the past research workers have assumed that k_2' (and by association, $1/\tau_2$) is not part of the H-S phase 2 in rabbit fibers (Abbott and Steiger, 1977; Goldman et al., 1987). This led to the previously mentioned anomaly that phase 2 in the frog has an apparent Q_{10} of 1.85, and the rabbit a Q_{10} of ~1.0. Earlier in the paper, we established that frog and rabbit fibers have a qualitatively similar four-phase response to a laser T-jump. In order to extend our observations on rabbit to frog fibers, we decided to examine the temperature dependence of the kinetics parameters obtained from laser T-jumps of frog fibers in greater detail. The key observation we made is that the temperature-sensitive reactions in both animals can be correlated once it is understood that their rates (specifically k_2' and $1/\tau_2$) scale with the working temperature of the muscle (frog ±25°C; rabbit 39.3°C) and not absolute temperature. The frog T-jump data shown in Fig. 3 were obtained at 3.5 and 13.5°C. Relaxation times obtained are $1/\tau_1 = 1840$ s^{-1}, $1/\tau_2 = 94$ s^{-1} and $1/\tau_3 = 8.5$ s^{-1} at 3.5°C for the data of Fig. 3, *A* and *B*; $1/\tau_2 = 133$ s^{-1} and $1/\tau_3 = 12.4$ s^{-1} at 8.5°C (data not shown) and $1/\tau_2 = 302$ s^{-1} and $1/\tau_3 = 9.7$ s^{-1} at 13.5°C for the data of Fig. 3, *C* and *D*. These individual relaxation times for the medium speed process ($1/\tau_2$) and other data resulted in an approximate Q_{10} of 2.6 for the process. There is no indication of a break in the kinetics similar to that seen with rabbit fibers justifying the comparison of the rabbit data obtained at and above 11°C with frog data recorded between 3.5° and 13.5°C. Data in the left-hand panel of Fig. 9 reveal that it is necessary to raise the temperature of rabbit fibers to 24°C in order to match in rate the temperature-dependent relaxation obtained with frog fibers at 13.5°C in the T-jump. The observation allows experiments in the two species to be correlated on a rational basis in which the reaction associated with k_2' and $1/\tau_2$ are equivalent and form part of phase 2. This assignment of the kinetics restores temperature sensitivity to phase 2 of the rabbit. $1/\tau_3$ in the frog, like the rabbit (Davis and Harrington, 1991) is unlikely to be a constituent of phase 2, because it is much slower and has a relaxation time that is temperature-independent (see data above).

Temperature dependence of T_2

The temperature dependence of T_2 is illustrated in Fig. 6. The extent of tension recovery normalized to the pre-L-step isometric tension remains more or less constant over the temperature range studied. This behavior contrasts with the increase seen in the T_1 values, plotted in the same Fig. 6.

DISCUSSION

T-jump kinetics in frog and rabbit fibers

Before considering the nature of the primary events of force generation it is important to establish the relationship be-

tween the kinetics of the H-S phases 1 and 2 of the frog and the T-jump and L-jump kinetics of rabbit psoas fibers. Our assignment of these reactions is quite different to that proposed hitherto (Abbott and Steiger, 1977; Goldman et al., 1987; Bershitsky and Tsaturyan, 1989). Here, we relate the experiments presented in the results section to the observations and interpretations of others.

Four kinetic phases are resolved in our T-jump experiments on rabbit and frog fibers. The first two steps consist of a drop in tension followed by partial recovery during a kinetically controlled relaxation ($1/\tau_1$) that follows. Goldman et al. (1987) first described and interpreted similar T-jump data as a transient drop in tension due to fiber expansion on heating followed by a virtually temperature-independent recovery of tension to a level above the pre-jump tension. This pair of steps was considered equivalent to a small L-jump. The conclusion was arrived at by comparing T-jump and L-jump data obtained under similar conditions. In general terms our data confirm both these findings and their interpretation. We find however, that the fast relaxation leads to only a partial recovery of initial tension (see Figs. 1–3). The analysis of the dominant biexponential process as a single exponential (see below) probably led to the overestimate of the amplitude of $1/\tau_1$ by Goldman et al. (1987). In our case the amplitude of the step drop in tension, as in earlier experiments (Davis and Harrington, 1987a), is smaller per unit temperature change than that observed by Goldman et al. (1987). Bershitsky and Tsaturyan (1989) point out that the infrared beam could have heated the fiber and its mountings in our experiments (Davis and Harrington, 1987a) and those of Goldman et al. (1987) causing the expansion of both and an artifactually large drop in tension. The interpretation is probably incorrect in our case since the mounting hardware is masked from the laser beam (Davis and Harrington, 1987b). In our T-jump experiments $1/\tau_1$ becomes increasingly difficult to resolve as temperature rises in both rabbit and frog fibers. This is partly expected because we find that the normalized amplitude of k'_1, (the equivalent L-jump relaxation) declines in amplitude with increasing temperature (see Fig. 7). We therefore chose to characterize the kinetics of this step in L-jump experiments. The overall response to a T-jump is completed in two additional kinetic steps. The elevated isometric tension after the T-jump arises almost entirely from large amplitude medium speed ($1/\tau_2$) and slow speed ($1/\tau_3$) relaxations (Davis and Harrington, 1987a). The relaxation rate constant of the medium speed process is temperature-dependent (see Fig. 8), while the slow relaxation is temperature-independent with an invariant $1/\tau_3$ value of $\sim 10\ s^{-1}$ in both frog and rabbit fibers (Davis and Harrington, 1991; Figs. 1–3). These two relaxations, unlike phase 1 and the fast component of phase 2, are the result of the direct effect of the T-jump on the reaction intermediates of the tension-generating cycle. Goldman et al. (1987) and Bershitsky and Tsaturyan (1988, 1989) did not resolve these relaxations in their T-jump experiments since they assumed that a monoexponential rise in tension gave rise to the isometric tension at the elevated post-jump temperature.

T-jump and L-jump kinetics compared

The correspondence of the low amplitude fast relaxation ($1/\tau_1$) seen in the T-jump to k'_1 of L-jump experiments was first investigated by Goldman et al. (1987) who considered this single kinetic step ($1/\tau_1$ or k'_1) equivalent to the complete H-S phase 2. A Q_{10} of 1.1 and rate constant of $400\ s^{-1}$ was assigned to the step. They were unable to reconcile their findings with the observations of Ford et al. (1977) who obtained a Q_{10} of 1.85 for the overall phase 2 in the frog. Our results indicate that this fast step is only a part of phase 2. In our experiments k'_1 increases from $950\ s^{-1}$ at 1°C with a Q_{10} of ~1.2 (tension per cross-bridge parallels the temperature rise and could conceivably contribute to the increase in rate). Abbott and Steiger (1977) were the first to report a Q_{10} of ~1.0 for this relaxation (K1 in their nomenclature) from their L-jump data.

We resolve the apparent paradox of the lack of a temperature-dependent phase 2 in the rabbit by including an extra kinetic component (k'_2 or $1/\tau_2$, in L-jump and T-jump experiments, respectively) that is temperature-sensitive and slower than k'_1. The inclusion of this well defined step with its Q_{10} of ~3.0 at temperatures above 11°C restores temperature sensitivity of the overall kinetics of phase 2, as first described in the frog (Ford et al., 1977). Various factors may have contributed to the exclusion of k'_2 from phase 2. Our T-jump experiments on rabbit and frog fibers show that $1/\tau_2$ scales with the working temperature of the muscle and not absolute temperature. This means that k'_2 and $1/\tau_2$ are slower in the rabbit than in the frog when the experiments are carried out at the same temperature. In the past, phase 2 has generally been considered a uniformly fast reaction lasting a brief 2–5 ms (Ford et al., 1977). At low temperatures in the rabbit, the slow component of phase 2 extends well beyond this limited time domain with a $t_{1/2}$ for the reaction as long as 12 ms. Ford et al. (1977) considered that each resolvable kinetic step in phase 2 responded in a similar manner to temperature; the presence of the temperature-dependent and temperature-independent steps was not anticipated. Abbott and Steiger (1977) resolved k'_2 (K2 in their nomenclature) but chose to assign it to cross-bridge attachment/detachment, rather than to a force generating structural change and thus, by definition, excluded it from phase 2 (their kinetics can not be compared with ours, because their activating solution contained 20 mM phosphate).

Physical properties of the reactions of phases 1 and 2

It is clear from the above discussion that phase 2 kinetics are comprised of two reactions with quite different physical properties. This brings into question the experimental basis of mechanisms in which the cross-bridge generates tension by stepping through a sequential set of like reactions of decreasing potential energy (Huxley and Simmons, 1971; Ford et al., 1977). The task in this section is to examine the heterogeneity of the kinetic steps that constitute phases 1 and 2

in an attempt to decide which are primary tension-generating transitions and which are not.

The temperature dependence of the kinetic amplitudes of phase 2 and of T_1 and T_2 concern us first. The relevant observation is that the component processes of phases 1 and 2 can readily be classified into two separate classes of reaction. Certain steps have relaxation amplitudes that scale with cross-bridge elasticity ($1 - T_1$ in Fig 6), while others scale with isometric tension. This provides a straightforward means of identifying and separating kinetic steps which result in de novo tension generation from those that arise from the mechanical perturbation of elastic or damped elastic elements within the cross-bridge. A requirement for this type of analysis is that the step under study must be kinetically uncoupled from adjacent kinetic steps. As noted in the Results, this kinetic constraint holds at temperatures between 6° and 26°C in rabbit fibers. This does not mean to say that the conclusions reached are invalid outside this temperature range; the constraints simply provide a "kinetic window" in which certain simplifying assumptions hold that allow the kinetics to be interpreted. Equilibrium coupling to slower steps in the steady-state actomyosin ATPase cycle is of little consequence, because it simply alters the number of force generating cross-bridges after phases 1 and 2 have equilibrated. Possible changes in the number of tension-generating crossbridges as the temperature is raised are corrected for by normalization to isometric tension at the temperature of the experiment. In conclusion, individual steps can be classified into tension-generating or elastic elements according to the temperature dependence of their amplitudes normalized to isometric tension (Figs. 6–9).

Amplitudes that scale with cross-bridge stiffness are considered first. The instantaneous stiffness data of phase 1 (see $1 - T_1$ response, Fig. 6) serves as a benchmark for the temperature dependence of an elastic element resident in the cross-bridge. This assignment is based on the observation of Ford et al. (1981) that at least 80% of the elasticity of phase 1 is located in the cross-bridge. The amplitude of the fast relaxation (k_1' and $1/\tau_1$) of phase 2 declines relative to the pre-jump isometric tension as temperature rises (Fig. 7), behavior similar in form to that observed for phase 1 (see T_1, Fig. 6). The similarity of the responses points to these phases being the product of elastic elements; one damped, the other not. Overall, the fast component of phase 2 recovery (k_1') serves to reverse the magnitude of the step-decline in tension of phase 1 thereby reducing the apparent stiffness from the high level seen at the extremity of phase 1 (T_1) to a lower value. Only strongly attached cross-bridges contribute to the L-jump and T-jump tension transients. Weakly attached cross-bridges are unlikely to contribute to the tension transient when perturbed by a L-jump of 160 μs duration at an ionic strength of 0.2 M (Brenner et al., 1982). The simplest interpretation of the results is that the fast step of phase 2 functions as a damped series elastic element in tension-generating cross-bridges. An alternative explanation exists if one assumes that strongly attached cross-bridges (particularly those attached prior to tension generation) are present in states that generate little or no tension (e.g. Ford et al., 1986; Brozovich et al., 1988). These bridges would be transiently loaded by a L-jump, producing a negative tension that would appear as an increase in the apparent stiffness recorded in phase 1. The time-dependent decay of tension, controlled by k_1', would arise from the dissociation of these strained bridges from actin resulting in a drop in fiber stiffness and a rise in tension.

Amplitudes that scale in fixed proportion to isometric tension at the temperature of the experiment are now considered. Tension recovery after the completion of phase 2 (T_2 response; Fig. 6) and the amplitude of temperature-dependent slow step of phase 2 (k_2' and $1/\tau_2$; Figs. 8 and 9) at temperatures of 6°C and above fall into this category. We therefore interpret these responses as direct manifestations of a primary tension-generating step. In T-jump experiments, virtually all the increase in isometric tension arises from two relaxations ($1/\tau_2$ and $1/\tau_3$). Of the two, $1/\tau_2$ is considered to be the primary force-generating step for the following reasons: The slow relaxation $1/\tau_3$, is excluded because it generates significant tension at a rate that does not change with temperature (Davis and Harrington, 1991 (Figs. 1 and 2)). Kinetics of this type are typical of a temperature-dependent rapid equilibrium (possibly ATP hydrolysis, see review by Kodama (1985)) prior to a rate-limiting step ($1/\tau_3$). A T-jump results in a step increase in the concentration of a reactant isolated from the tension-generating step by a rate-limiting step; the slow interconversion of reactant to product follows via the temperature-insensitive rate-limiting step. Rapid interconversion of product into the tension-generating intermediate follows tracking the kinetics of the rate-limiting step. This leaves $1/\tau_2$, the slow step of phase 2, as sole candidate for the primary tension-generating step. Support for this assignment comes from the correspondence of $1/\tau_2$ and k_2' and from the observation that the slowest kinetic component of phase 2 in the frog is unique to actively contracting fibers (Jung et al., 1988). The step is clearly endothermic since a T-jump results in a marked rise in fiber tension and the relaxation rate constant changes with temperature (Fig. 9). The following observations are consistent with the above interpretation. Ford et al. (1977), found that tension recovery during phase 2 was temperature-dependent in frog fibers. Earlier T-jump experiments of ours on rigor and activated fibers showed that a fast kinetic step in the time domain of phase 2 is endothermic (Davis and Harrington, 1987a). More importantly, direct calorimetric evidence for heat absorption in the time domain of phase 2 in frog fibers was obtained by Gilbert and Ford (1988). We therefore conclude that a single endothermic step in the time domain of phase 2 is responsible for tension generation in rabbit and frog fibers. Slower relaxations (e.g., $1/\tau_3$) generate tension by being coupled to the primary force-generating event.

Nature of the tension-generating endothermic reaction

The endothermic character of the slow step of phase 2 implies that tension generation is entropy driven. This correlation rests on fundamental thermodynamic principles: a negative

free energy for a reaction with a positive ΔH will only result if there is a compensating and sufficient increase in entropy to drive the transition. An order-disorder transition therefore appears to be associated with tension generation in muscle.

The experiments described in the paper are specifically concerned with determining the physical nature of the tension-generating step and do not directly address the issue of the location of the order-disorder transition within the cross-bridge. In vitro motility assays have shown that the interaction of the myosin head and actin alone can generate tension (Kishino and Yanagida, 1988; Huxley, 1990). The amount of tension generated per S1 in the in vitro motility assays is lower (three- to sixfold) than in muscle fibers. It is unclear at this point in time whether the observed tension deficit is real or whether it is a consequence of the assay. This ambiguity in the interpretation leaves open the possibility that tension generation could occur at various sites in the cross-bridge, resulting in a hybrid mechanism of tension generation. A hybrid mechanism is however at odds with our observation that a single-step endothermic transition appears to generate tension in muscle under isometric conditions. A correlation of structural changes in the cross-bridge during the time course of the slow component of phase 2 is required in order to determine whether the simplest conclusion, that the endothermic tension-generating step is located in the acto-S1 complex, is correct or not.

Two different types of structural change in proteins can give rise to entropy-driven reactions capable of generating tension. The first class of mechanism can be considered akin to protein self-assembly in which the order-disorder transition is the result of the exclusion of structured water from the interacting surfaces of the protein. The formation of the actomyosin complex provides a good example of this type of reaction (see Kodama, 1985), the rotation of the subunits of the hemoglobin tetramer relative to one another during the R to T transition is yet another. In this context, Geeves et al. (1984) have proposed models in which the weak to strong transition in the binding of myosin to actin is linked either directly or indirectly to tension generation. Direct coupling operates in the mechanism of Huxley and Simmons (1971) in which the myosin head generates tension while stepping through a series of like binding sites of decreasing potential energy on actin. Our observation that tension generation occurs in a single step makes mechanisms like this in which Brownian (thermal) motion provides the activation energy for transitions between a series of binding sites of declining potential energy less plausible. The second class of mechanism are characterized by reactions in which organized structure of low entropy is converted into freely jointed random-coil of high conformational entropy that generates tension; a process akin to protein denaturation. The proposed helix-coil mechanism of muscle contraction is a well documented example of this type of mechanism (see Harrington et al., 1990). The main physical feature that can be used to distinguish between the two mechanisms is that, in denaturation-type reactions, tension generation is accompanied by a decrease in stiffness resulting from the interconversion of the relatively rigid organized structure of the pre-tension-generating state into more compliant tension-generating random-coil. The stiffness of cross-bridges already in the tension-generating state (random-coil) should increase slightly in proportion to absolute temperature. Additional experiments are needed in order to determine whether the stiffness during phase 1 or of the fast component of phase 2 changes in the manner described as temperature is used to bias the equilibrium toward tension generation. A recent report of Bershitsky and Tsaturyan (1989) on stiffness changes following a T-jump is interpreted to show that stiffness does not change during tension generation. They conclude from their kinetic data that tension in muscle is not generated by an order-disorder transition involving the formation of random-coil. As we have shown, the analysis of their T-jump relaxation spectra appears incomplete. They are therefore unable to assign changes in stiffness to specific cross-bridge events such as tension generation or the interconversion of weak and strongly bound cross-bridges. In a recently published paper they resolved two rate constants ranging from 450–1750 s^{-1} and 60–250 s^{-1} with similar Q_{10} values of 1.7 and 1.8, respectively (Bershitsky and Tsaturyan, 1992). As with their earlier experiments, these large perturbation relaxation experiments are not directly comparable with the quite different rate data obtained from the small perturbation relaxation experiments described in this paper. Large perturbation relaxation experiments result in a significant change in the flux of reactants through the overall actomyosin ATPase cycle making the interpretation difficult (for example steps after the rate limiting step will not be observed). Small perturbation relaxation experiments allow the independent equilibration and resolution of individual steps before and after the rate-limiting step with a much reduced change in the flux of reactants through the overall reaction (see Bernasconi, 1976).

Under the conditions of our experiments the distance over which the crossbridge acts (the throw) appears independent of temperature. The pertinent observation is that the T_2 (the extent of recovery tension during phase 2) and the amplitude of the tension-generating step associated with k_2' remains a fixed percentage of isometric tension independent of temperature (Figs. 6 and 8). Tension generation in isometric fibers appears to arise from the contraction of an element of finite size. This type of response would correlate with tension generation by well defined interactions between organized domains of a protein or by the melting of a segment of protein of finite size. Additional experiments with different sized step changes in length are necessary to confirm the observation.

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