Crossbridge Scheme and the Kinetic Constants of Elementary Steps Deducible From Chemically Skinned Papillary and Trabecular Muscles of the Ferret

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Elementary steps of the crossbridge cycle in chemically skinned ferret myocardium were investigated with sinusoidal analysis. The muscle preparations were activated at pCa 4.82 and an ionic strength of 200 mM, and the effects of the change in the MgATP (S) and phosphate (Pi) concentrations on three exponential processes were studied at 20°C. Results are consistent with the following crossbridge scheme:

\[
\begin{align*}
AM & \xrightleftharpoons[\text{S}]{k_{1a}} AM+S & \xrightleftharpoons[k_{1b}]{} AM^\ast S & \rightleftharpoons[k_{-1b}]{} \text{Det} & \rightleftharpoons[k_4]{} AM^\ast DP & \xrightleftharpoons[k_5]{} P & \xrightarrow[k_6]{} AM^\ast D
\end{align*}
\]

where A is actin, M is myosin, D is MgADP, and Det includes all detached states (MS and MDP). From our studies, we obtained \(K_{1a}=0.99\;\text{mM}^{-1}\) (MgATP association), \(k_{1b}=270\;\text{s}^{-1}\) (ATP isomerization), \(k_{-1b}=280\;\text{s}^{-1}\) (reverse isomerization), \(K_{1b}=k_{1b}/k_{-1b}=0.95\), \(k_2=48\;\text{s}^{-1}\) (crossbridge detachment), \(k_3=14\;\text{s}^{-1}\) (reverse detachment), \(K_1=3.5\), \(K_4=11\;\text{s}^{-1}\) (crossbridge attachment), \(k_4=107\;\text{s}^{-1}\) (reverse attachment), \(K_5=0.11\), and \(K_6=0.06\;\text{mM}^{-1}\) (Pi association). \(k_6\) is the rate-limiting step, and it is the slowest forward reaction in the cycle, which results in the rigidlike AM state. \(K_{1a}\) (MgATP binding) is four times that of rabbit psoas, and \(K_5\) (Pi binding) is 0.3 times that of psoas, demonstrating that crossbridges in myocardium bind MgATP more and Pi less than psoas. The rate constants of ATP isomerization \((k_{1b}, k_{-1b})\), crossbridge detachment \((k_2, k_3)\), and crossbridge attachment \((k_4)\) steps are generally an order of magnitude slower than rabbit psoas. The reverse attachment step \((k_{-4})\) is similar to that in psoas, indicating that this step may occur irrespective of the myosin type and possibly spontaneously. The above scheme with the deduced kinetic constants predicts the following crossbridge distributions at 5 mM MgATP and 8 mM Pi: AM (3%), AM+S (15%), AM^\ast S (14%), Det (50%), AM^\ast DP (6%), and AM^\ast D (12%). The actual number of attached crossbridges was measured to be 51±4% by the stiffness ratio during activation and after rigor induction, and a strong correlation was seen with the prediction. Our results are consistent with the hypothesis that force generation occurs at the Det→AM^\ast DPi transition, and the same force is maintained after the release of Pi. (Circulation Research 1993;73:35-50)

KEY WORDS • myocardium • crossbridge scheme • rate constant • elementary step • phosphate • MgATP

It is generally believed that the cyclic interaction of myosin crossbridges with actin on thin filaments is responsible for force generation and shortening in muscle. It is also believed that this interaction is driven by the free energy of ATP hydrolysis and the system's ability to absorb the products of hydrolysis (MgADP, phosphate [Pi], H^+, and work output). The interaction between actin, myosin, ATP, and its hydrolysis products has been studied extensively using biochemical techniques on isolated muscle proteins, and numerous intermediate states have been identified. However, the most important aspect of contractility—force generation—could not be learned with these solution studies. Also, we could not determine the modulation of the crossbridge cycle by imposed length change. It has long been thought that the strain on crossbridges modifies the rate constants of elementary steps in the crossbridge cycle. To circumvent these shortfalls, investigators have used single muscle fibers mostly from skeletal sources and applied mechanical perturbations to characterize...
the elementary steps of the crossbridge cycle. These studies on intact\textsuperscript{2,3} and skinned\textsuperscript{10,14} preparations have suggested that there are a number of intermediate states and distinct transitions between the states involved in the cyclic interaction of crossbridges with actin sites. Skinned fibers are advantageous for these studies, because one can apply chemical perturbations as well as mechanical perturbations, so that parallel experiments with solution studies are possible. The correlation between various states of the crossbridges implied by the mechanical measurements and those observed in biochemical experiments has been discussed.\textsuperscript{5,12,25} Until recently, however, the detailed elementary steps in muscle fibers have not been elucidated, primarily because of the complexity of the system needed to solve the elementary steps.\textsuperscript{15}

With improved techniques and simplified thinking, we have solved the elementary steps of the crossbridge cycle in fast-twitch skeletal muscle fibers.\textsuperscript{13,16} Our approach is to change the length of a muscle preparation sinusoidally at varying frequencies ranging up to the orders of magnitude and to detect tension time courses. The length change presumably modifies the rate constants of the elementary steps, and this modification results in a transition between the crossbridge states. Since most crossbridge states support tension and some do not, the transition is sensed as "tension transients" in the time domain analysis\textsuperscript{6,17} and "exponential processes" in the frequency domain analysis.\textsuperscript{18,19} With the sinusoidal analysis technique, a chemical reaction is resonated to the length oscillation of a particular frequency, and this resonance is detected by amplitude and phase shift in tension. In sinusoidal analysis, it is approximately correct to state that a reaction faster than the frequency of oscillation appears to be at equilibrium, whereas a reaction slower than the frequency of oscillation appears not to happen. By selecting a particular frequency, one can study a specific chemical step (elementary step) in the crossbridge cycle.

However, systematic studies on cardiac muscles have lagged behind those on skeletal muscles, and many of the cardiac studies to date have been focused on a description of the results rather than finding the fundamental mechanisms of contraction. This is due to a paucity of cardiac muscle specimens and difficulty in their preparation as well as the general complexity of the crossbridge cycle. The results from cardiac muscles are more difficult to interpret because of greater end compliance and parallel elasticity than in skeletal muscles. In principle, techniques developed in skeletal muscles can be applied to studies in myocardial crossbridge dynamics.\textsuperscript{20-26} Since we were successful in elucidating the elementary steps in the crossbridge cycle in fast-twitch rabbit skeletal muscles, we are now applying the sinusoidal analysis technique to mammalian heart muscles.\textsuperscript{27-29}

We have previously studied the mechanical response of ferret myocardium, and identified three exponential processes (B, C, and D in Reference 27) in the complex modulus of maximally activated intact and skinned preparations. In elucidating the elementary steps of the crossbridge cycle, it is essential to study the effect of MgATP and Pi concentrations on the rate constants of exponential processes in skinned fibers. This is because most parameters needed to characterize the crossbridge cycle under physiological conditions can be obtained from these studies. In this report, we study the effects of MgATP and Pi on the exponential processes and isotropic tension in chemically skinned ferret papillary and trabecular muscles. This preparation is advantageous because of easy dissection, and it primarily consists of the V_{2} (β) myosin isoform;\textsuperscript{26} hence, the kinetics are expected to be homogeneous. One purpose of this report is to construct a crossbridge scheme in myocardium and to measure the rate and association constants that characterize the elementary steps. Another purpose is to compare our results on myocardium with those on fast-twitch skeletal muscle fibers\textsuperscript{30} and to correlate our results with corresponding rate constants known from solution studies\textsuperscript{31-34} where possible.

### Materials and Methods

**Chemicals and Solutions**

H\textsubscript{2}EGTA, Na\textsubscript{2}H\textsubscript{2}ATP, Na\textsubscript{2}creatine phosphate (CP), KH\textsubscript{2}PO\textsubscript{4}, K\textsubscript{2}HPO\textsubscript{4}, 3H\textsubscript{2}O, glucose, MOPS, Na\textsubscript{2}N\textsubscript{2}, and glutaraldehyde were purchased from Sigma Chemical Co, St Louis, Mo; CaCO\textsubscript{3}, KOH, CaCl\textsubscript{2}, 2H\textsubscript{2}O, KCl, MgCl\textsubscript{2}, 6H\textsubscript{2}O, Na\textsubscript{2}HPO\textsubscript{4}, H\textsubscript{2}O, MgO, NaCl, NaOH, propionic (Prop) acid, and Triton X-100 were from Fisher Scientific Co, Itasca, Ill. Creatine kinase (CK) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind, and NaHCO\textsubscript{3} was from EM Science, Cherry Hill, NJ. All chemicals were of analytical grade.

The Tyrode's solution used for dissecting intact myocardium contained (mM): NaCl, 130; CaCl\textsubscript{2}, 2.5; KCl, 4; MgCl\textsubscript{2}, 1; Na\textsubscript{2}HPO\textsubscript{4}, 0.435; NaHCO\textsubscript{3}, 10; and glucose, 5.6. The solution was continuously oxygenated with a gas mixture (98% O\textsubscript{2}-2% CO\textsubscript{2}). When equilibrated with this gas mixture, the Tyrode's solution attained a P\textsubscript{O} of 540 to 570 mm Hg and a pH of 7.4.\textsuperscript{23} The chemical skinning solution contained (mM): K\textsubscript{2}H\textsubscript{2}EGTA (EGTA), 5; Na\textsubscript{2}MgATP (MgATP), 2; Na\textsubscript{2}K\textsubscript{2}H\textsubscript{2}ATP (free ATP), 5; NaProp, 130; and imidazole, 6; along with 1% (vol/vol) Triton X-100.

To compare the present results with those in our previous studies on rabbit psoas fibers, we used the same solutions as in our previous studies,\textsuperscript{13,16,25} except that, in myocardial activating solutions, 10 mM NaProp was replaced with 10 mM NaN\textsubscript{2}. NaN\textsubscript{2} was used to inhibit the ATPase of mitochondria, which are abundantly present in myocytes. The relaxing solution contained (mM): EGTA, 6; MgATP, 2; free ATP, 5; K\textsubscript{2}H\textsubscript{2}PO\textsubscript{4} (Pi), 8; KProp, 48; NaProp, 62; and MOPS, 10. The wash solution contained (mM): MgATP, 0.3; Pi, 8; KProp, 103; NaProp, 75; and MOPS, 10. The control activating solution contained (mM): K\textsubscript{2}CaEGTA (CaEGTA), 6; MgATP, 5.3; free ATP, 4.7; CP, 15; Pi, 8; NaProp, 8-28; KProp, 30-40; NaN\textsubscript{2}, 10; and MOPS, 10; along with 160 U/mL CK. The experimental solution that changed MgATP concentrations contained (mM): CaEGTA, 6; MgATP, 0-10.6; free ATP, 4.4-5.0; CP, 15; Pi, 8; NaProp, 8-28; KProp, 30-40; NaN\textsubscript{2}, 10; and MOPS, 10; along with 160 U/mL CK. When the MgATP effect is plotted, the concentration of MgATP\textsuperscript{52} is shown in the abscissa, because this ionic species is known to be the substrate of the actomyosin system.\textsuperscript{1} The solution that changed Pi concentrations contained (mM): CaEGTA, 6; Pi, 0-16; MgATP, 5.3; free ATP,
preparation. The sarcomere length, determined by optical diffraction using a helium-neon laser, ranged from 2.2 to 2.3 \( \mu \)m, which is consistent with earlier works.\textsuperscript{36,37}

**Experimental Procedure**

All experiments were carried out during maximal \( \text{Ca}^{2+} \) activation at 20°C. Methods of obtaining the complex modulus data and of extracting the apparent rate constants of exponential processes were described in our earlier works.\textsuperscript{12,18} In brief, we changed \( L_0 \) sinusoidally with small amplitudes (0.25% \( L_0 \) peak to peak corresponding to \( \pm 1.4 \) \( \mu \)m per crossbridge) and detected tension amplitude and phase shift at 19 discrete frequencies ranging from 0.13 to 135 Hz. This frequency range corresponds to 1.2 to 1200 milliseconds in the time domain. The duration of the data collection was 29 seconds. Both length and tension signals were filtered by first-order low-pass filters (cutoff, 150 kHz) and simultaneously digitized by two 15-bit analog-to-digital converters at the rate of 100 kHz. At the beginning of each frequency of length oscillation, the data were not sampled for 0.25 seconds to wait for the steady state. Thereafter, the data were continuously sampled and accumulated in two time courses (length and tension); each comprise 40 points that represent one cycle (period). The data were collected for 0.4 seconds (or for a convenient time at which a cycle is complete in excess of 0.4 seconds) at each frequency. Our method of data collection eliminates the aliasing problem in all frequencies used. Before and after the length oscillation at each frequency, isometric tension levels were measured after the steady state was reached in 0.25 seconds, and the tension time course was detrended on the basis of these measurements. (This procedure was usually not necessary, because isometric tension was adequately stable during the measurement.) These operations were controlled by a Nova 4S computer (Data General Corp., Southborough, Mass.) with a 40-kilobyte memory capacity for program and data storage and by a homemade analog/digital interface named PEACh. While waiting for the steady-state, the real (in phase) and imaginary (quadrature) parts of the first-order terms of the Fourier expansion were calculated by multiplying sine and cosine functions to two time courses followed by integration, and the complex modulus data \( Y(f) \) were calculated by the ratio of the two first-order terms: \( Y(f) \) is the ratio of the stress (force per unit area) change to the strain (relative length) change expressed in the frequency \( f \) domain. An advantage of our method is that a particular reaction step can be made to resonate to the imposed length oscillations, thus maximizing the signal from the reaction step. This method is intrinsically a signal-averaging procedure; hence, the signal from the reaction step can be enhanced by increasing the duration of the measurement. The data were corrected for system response by using the complex modulus of fixed myocardium with 2.5% glutaraldehyde after high rigor state\textsuperscript{38} was induced.

In a typical experiment, a preparation was placed in the relaxing solution (1 mL); then the solution was changed with the wash solution to remove EGTA. This was followed by two full volume changes of the experimental solution, but without CaEGTA. Complex modulus data were collected to record the response from the relaxed myocardium, and 100 \( \mu \)L of 66 mM CaEGTA in

**Muscle Preparations**

Male ferrets (\( \geq 800 \) g in body weight) were anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg), hearts were removed quickly, and papillary and trabecular muscles from the right ventricle were excised in a Petri dish that contained oxygenated Tyrode's solution. Each end of the excised myocardium was tied by a silk thread to a small hook made of tungsten wire (125 \( \mu \)m in diameter). Similar hooks were inserted into the moving ends of a length driver and a tension transducer for the muscle attachment. The muscle preparation was then suspended horizontally between the two hooks. The length driver and the tension transducer were described in our earlier report.\textsuperscript{18} The experimental chamber likewise contained oxygenated and transfused Tyrode's solution, in which the muscle preparation was suspended. The muscle was electrically stimulated with a pulse of 5-millisecond duration and a voltage of 20% to 50% above the threshold level via a pair of platinum plate electrodes that were 7 mm apart and mounted alongside the preparation. The preparation was stabilized by repetitive stimuli at the rate of 12 per minute until the steady twitch tension was observed. Then, the muscle length (\( L_0 \)) was adjusted to give the maximum twitch tension. The preparation was subsequently placed in the \( \text{Ca}^{2+} \)-free Tyrode's solution. After the twitch contraction almost disappeared (=20 minutes), the muscle was treated with the chemical skinning solution for 60 minutes. The solution was constantly stirred by a vibrator to maximize the thinning. The skinned muscle was then bathed in the relaxing solution (1 mL). At this point, the diameter of the preparation was determined with an ocular micrometer, and the cross-sectional area was estimated. The diameter ranged from 130 to 560 \( \mu \)m, and \( L_0 \) ranged from 1.5 to 3.9 mm. These values were used to calculate the tension and complex modulus of the muscle.
10 mM MOPS (pH 7.00) was added. The change in pH was negligible (±0.01). The tension started to rise and plateaued within 20 to 30 seconds. The complex modulus data were then collected for the active response. The preparation was subsequently relaxed with two full volume changes with the relaxing solution. This procedure was repeated for the control activating solution and for a series of solutions that changed the MgATP or Pi concentration. These studies were carried out both in increasing and decreasing orders of MgATP or Pi concentrations so that any artifact due to the order of experiments (if such existed) was canceled after averaging. The complex modulus collected during relaxation was usually subtracted from that of Ca^{2+} activation. The complex modulus of relaxed myocardium is much higher than that of skeletal muscles at their optimal lengths; the relaxed modulus represents contributions from elements that are mechanically in parallel with crossovers, such as collagen, elastin, cell membrane, and connectin-titin. An advantage of our method is that these elements do not affect the kinetic measurements of crossovers.

**Results**

**Effects of MgATP on Complex Modulus and Isolation of Exponential Processes**

Skinned preparations were activated with a series of MgATP solutions and the complex modulus data \(Y(f)\) were collected at 19 discrete frequencies \((f)\). Fig 1 shows the result plotted in the dynamic modulus versus frequency (panel A), and in the phase shift versus frequency (panel B) at three different MgATP concentrations. The dynamic modulus is defined as the length of the \(Y(f)\) vector and the phase shift is defined by the direction (angle from the abscissa) of the \(Y(f)\) vector. For the purpose of display, the relaxed complex modulus was not subtracted from the data shown in Fig 1 following earlier conventions. As seen in Fig 1A, an increase in the MgATP concentration shifts the dynamic modulus versus frequency plot to the right. In Fig 1B, it is seen that the increase in the MgATP concentration similarly shifts the phase versus frequency plot to the right. These results imply that crossbridge kinetics became faster at higher MgATP concentrations. Figs 1A and 1B further demonstrate that this speeding effect of MgATP was significant when the concentration was increased from 0.1 to 1 mM, but the effect was almost saturated for further increases to 10 mM. Fig 1C is the Nyquist plot of the same data, which is the plot of the viscous modulus versus elastic modulus. The viscous modulus is the 90° out of phase (quadrature) component of \(Y(f)\), and the elastic modulus is the in-phase component of \(Y(f)\). Nyquist plot is convenient for the identification of exponential processes, because one hemicircle (half circle) represents one exponential process. Since three hemicircles can be identified in Fig 1C, we assume that \(Y(f)\) can be resolved into three exponential processes—B, C, and D—in the order of increasing speed:

\[
Y(f) = H - B/(1 + b/f) + C/(1 + c/f) + D/(1 + d/f)
\]

**FIG 1.** The effect of MgATP (0.1, 1, and 10 mM) on the complex modulus \(Y(f)\) of ferret papillary muscle is plotted in dynamic modulus \([Y(f)]\) vs. frequency in panel A, in phase shift \([\text{Arg } Y(f)]\) vs. frequency in panel B, and in viscous modulus \([\text{Imag } Y(f)]\) vs. elastic modulus \([\text{Real } Y(f)]\) in panel C (Nyquist plot). The phosphate concentration was fixed to 8 mM. Averaged results are from nine experiments. The frequencies used are 0.13, 0.25, 0.35, 0.5, 0.7, 1.0, 1.4, 2.0, 3.2, 5.0, 7.5, 11, 17, 25, 35, 50, 70, 100, and 135 Hz. In the Nyquist plot (panel C), the low-frequency end is near the origin, and the high-frequency end is to the right of the plot. Curved lines indicate theoretical projections based on Equation 1 and the best-fit parameters. Exponential processes B, C, and D are indicated in panel C. \(f_{\text{max}}\) is defined as the frequency that gives the minimum dynamic modulus in panel A, and \(f_{\text{max}}\) is defined as the frequency that gives the maximum phase shift in panel B. For the purpose of display, the resting complex modulus was not subtracted from the data.
where \( i = \sqrt{-1} \). The three processes are marked in Fig 1C as B, C, and D. Lowercase letters \( b, c, \) and \( d \) \((b < c < d)\) represent characteristic frequencies of exponential processes B, C, and D, respectively; uppercase letters \( B, C, \) and \( D \) represent their respective magnitudes. Characteristic frequencies multiplied by \( 2\pi \) are the apparent (measured) rate constants. \( H \) is the elastic modulus extrapolated to zero (0) frequency. We also define the elastic modulus extrapolated to the infinite (*) frequency:

\[
Y_* = H - B + C + D
\]

\( Y_* \) corresponds to phase 1 of step analysis\(^5,17\) and is proportional to both the number of attached crossbridges and the stiffness of each crossbridge after a proper subtraction of the relaxed modulus. For this reason, we refer to \( Y_* \) as stiffness, and this nomenclature is used throughout this report. Process D corresponds to the fast component of phase 2; process C, to the slow component of phase 2; and process B, to phase 3 of step analysis. Process B is also known as the oscillatory work component\(^19,40,41\) in sinusoidal analysis or delayed tension\(^20,25,42\) in step analysis. We found that, in activated myocardium, a slow process (A) was either very small or absent; hence, we did not include this term in Equation 1. Process A is normally present in fast-twitch skeletal muscles\(^18,43\) and corresponds to phase 4 of step analysis.

It is seen in Fig 1A that the dynamic modulus assumes the minimum value at around 1 to 2 Hz; hence, this frequency is called \( f_{\text{min}} \).\(^21,39\) In myocardium, we find that \( f_{\text{min}} \) approximates the characteristic frequency \( b \).\(^27\) It is seen in Fig 1B that the phase shift assumes the maximum value at 2 to 3 Hz; hence, we define this frequency as \( f_{\text{max}} \). In both rabbit psoas and ferret myocardium, \( f_{\text{max}} \) approximates the characteristic frequency \( c \). There is no convenient approximation for the characteristic frequency \( d \), but the presence of process D is apparent at the high frequency end of the Nyquist plot at the branch labeled D (Fig 1C). Except for our recent report\(^27\), the process that corresponds to process D has not been identified in myocardium; this may be due to the lower frequency range used (<60 Hz) in earlier studies.\(^21,24,39,42\) In our experience, process D is more difficult to characterize than process B or C, because magnitude \( D \) is 1/2 to 1/4 of magnitude \( C \) and because the characteristic frequency \( d \) is close to the high frequency end where the data are subject of increased artifacts, such as those caused by the mass of the hooks used to mount the preparation. In both skeletal\(^16,18\) and cardiac\(^27\) muscle preparations, all exponential processes are absent when muscle fibers are relaxed (no Ca\(^{2+}\)) or brought into the rigor condition (no MgATP); hence, they are considered to reflect the dynamic interaction of crossbridges with the thin filaments.

As is evident in Figs 1A and 1B, both \( f_{\text{min}} \) and \( f_{\text{max}} \) increase with an increase in the MgATP concentration, suggesting that \( 2\pi b \) and \( 2\pi c \) both increase with the MgATP concentration. It is further seen from this figure that the increase is evident when the MgATP concentration is raised from 0.1 to 1 mM, but this effect appears to saturate for further increase. The complex modulus data were fitted to Equation 1, and the apparent rate constants and their magnitude parameters were extracted. The theoretical projections based on Equation 1 and the fitted parameters are plotted by the curved lines in Fig 1. As seen in this figure, Equation 1 describes the complex modulus of activated myocardium adequately. The fit is somewhat off in the low-frequency range (0.1 to 1 Hz) in the phase-shift plot (Fig 1B) because the phase shift is less reliable at lower frequencies where the dynamic modulus is smaller (Fig 1A). We minimized the sum of squared deviations in the complex plane\(^18\) (Nyquist plot), and consequently, the error is distributed homogeneously in the Nyquist plot (Fig 1C) and in the dynamic modulus plot (Fig 1A).

**Effect of MgATP on Rate Constants, Tension, and Stiffness (\( Y_* \))**

The apparent rate constants \( 2\pi b, 2\pi c, \) and \( 2\pi d \) were averaged for five or six experiments and plotted against the MgATP concentration in Fig 2. As seen in this figure, all three rate constants increased when the MgATP concentration was increased from 0.1 to 2 mM but the effect was almost saturated for further increase in the MgATP concentration. The effect was largest with \( 2\pi d \), but all three rate constants were similarly affected (Fig 2A). The effect of MgATP on \( 2\pi b, 2\pi c, \) and \( 2\pi d \) is consistent with our earlier reports on rabbit psoas fibers,\(^12,16,35\) except that the rate constants are generally an order of magnitude smaller in myocardium than in psoas.

Fig 3A represents isometric tension plotted against the MgATP concentration for the same experiments. As seen in this figure, the tension decreased with an increase in the MgATP concentration from 0.1 to 2 mM, and the tension was saturated for further increase in the MgATP concentration. The effect of MgATP on isometric tension is consistent with earlier reports on skeletal,\(^35,42\) insect,\(^41,44\) and cardiac\(^45-48\) muscle preparations. Fig 3B represents stiffness (\( Y_* \)) plotted against the MgATP concentration. This plot is generally similar to the tension versus MgATP plot of Fig 3A. \( Y_* \) decreased gradually as the MgATP concentration was increased from 0.1 to 2 mM, indicating that fewer crossbridges were attached at higher MgATP concentrations. This effect was saturated above 2 mM. To examine if the MgATP dependence of isometric tension and stiffness is different, the ratio of tension to \( Y_* \) is plotted in Fig 3C. This figure demonstrates that the ratio increased as the MgATP concentration was increased. The increase was small but significant; most of the error bars are smaller than the symbol size and cannot be seen. The increase in the ratio of tension to \( Y_* \) implies that crossbridges with the lower force state(s) were converted to the higher force state(s) as the MgATP concentration was increased. The ratio represents the averaged force per crossbridge, and it is also the amount of length release required for abolishing full tension during contraction.

**Effect of Pi on Complex Modulus**

Fig 4 represents the complex modulus plotted at three different Pi concentrations. The same plotting conventions are used as in Fig 1. As is evident in Figs 4A and 4B, both \( f_{\text{min}} \) and \( f_{\text{max}} \) increased with an increase in the Pi concentration, suggesting that \( 2\pi b \) and \( 2\pi c \) both increased. This situation is different from rabbit psoas fibers, where only \( 2\pi b \) increased with Pi and \( 2\pi c \) did not change much with Pi.\(^49\) In Figs 4A and 4B, the effect of
Pi was significant when increased from 0 to 8 mM, but the effect was smaller for a further increase to 16 mM. The complex modulus data were fitted to Equation 1, and the apparent rate constants (2πb, 2πc, and 2πd) and their magnitude parameters were extracted. The theoretical projection based on Equation 1 and the fitted parameters are plotted by the curved lines in Fig 4. As seen in this figure, the fit was satisfactory. However, as explained in the case of the MgATP effect (Fig 1B), the fit was somewhat off in the low-frequency range in the phase-shift plot (Fig 4B).

**Effect of Pi on Rate Constants, Tension, and Y.**

The apparent rate constants were averaged for eight experiments and plotted against the Pi concentration in Fig 5. It is seen from this figure that the apparent rate constants 2πb and 2πc increased with an increase in the Pi concentration, and the plots are concave downward; thus, the effect of Pi is larger at lower concentrations.

The effect of Pi on 2πd was smaller or absent (Figs 5A and 5D); the amount of the increase in 2πd was 1.4-fold when the averaged values were compared between 0 and 16 mM. This contrasts with a 2.3- to 2.5-fold increase in 2πb and 2πc. The SEM is generally larger with 2πd because process D was more difficult to characterize than process B or C. The small effect on 2πd could be due to the large effect of Pi on 2πc, which may have interfered with the data-fitting procedure to find 2πd. Therefore, we focus on the effect of Pi on

**Fig 3.** Isometric tension (in panel A), stiffness (Y, in panel B), and the tension (T) to Y ratio (in panel C) are plotted against the MgATP concentration. The phosphate concentration was 8 mM. The resting tension and stiffness was subtracted first, the data were normalized to the control condition (5 mM MgATP), and then averaging was performed over eight experiments and plotted with SEM bars. The error bars smaller than the symbol size cannot be seen. Data points are connected by straight lines. After subtraction of resting values, tension in the 5 mM MgATP solution averaged 18±3 kN/m², and Y averaged 0.89±0.20 MN/m² (mean±SEM, n=8).
FIG 4. Graphs showing the effect of phosphate (Pi, 0, 8, and 16 mM) on the complex modulus Y(ω) of ferret papillary muscle. The MgATP$^{-}$ concentration was fixed to 5 mM. Averaging was performed over eight experiments. The same plotting conventions as in Fig 1 are used. B, C, and D in panel C indicate the three exponential processes.

processes B and C in this report. The large effect of Pi on $2\pi b$ is similar to that observed on rabbit psoas fibers,$^{13,48}$ except that the rate constants are generally an order of magnitude smaller in myocardium than in psoas. The Pi effect on $2\pi b$ is generally consistent with that on the reciprocal time constant of the delayed rise in tension (phase 3) in cardiac muscles.$^{20}$ The rate constant $2\pi b$ is identical to the reciprocal of the time constant of phase 3, and the characteristic frequency $b$ is similar to the optimal frequency of oscillatory work.$^{18}$

Fig 6A represents isometric tension plotted against Pi concentration for the same experiments. As seen in this figure, the tension decreased gradually with an increase in the Pi concentration, and the plot was concave upward. The effect of Pi on isometric tension is consistent with earlier reports on skeletal,$^{10,13,49,50,52}$ insect,$^{53}$ and cardiac$^{26,52,54,55}$ muscles. We did not observe a correlation between the diameter of the preparation and isometric tension at high Pi concentration as observed by Kentish,$^{55}$ possibly because we used a higher concentration of CK (160 U/mL) than that used by Kentish (2.5 to 3.8 U/mL); other experimental conditions were similar. It is likely that the diameter of the preparation becomes a critical factor when the CK concentration is low.

Fig 6B represents stiffness ($Y_\gamma$) plotted against Pi concentration. This plot is generally similar to the tension versus Pi plot of Fig 6A. $Y_\gamma$ decreased gradually as the Pi concentration was increased, indicating that fewer crossbridges were attached at the higher Pi concentration. To examine the Pi dependence of isometric tension and stiffness differ, the ratio of tension to $Y_\gamma$ is plotted in Fig 6C. This figure demonstrates that the ratio decreased slightly as the Pi concentration was increased. This implies that at higher Pi concentrations, crossbridge populations were shifted to the state(s) that supports less tension.

**Crossbridge Scheme**

To interpret our results, we used the crossbridge scheme developed by sinusoidal analysis using skinned psoas fibers of the rabbit.$^{13,16,56}$ This scheme has seven crossbridge states, and it is characterized by seven rate constants and three association constants (called kinetic constants) that govern transitions between the states. This scheme is depicted in Fig 7 and the abbreviations are defined as follows: A, actin; M, myosin head; S, MgATP; D, MgADP; and F, Pi (phosphate). S, P, and D also indicate respective concentrations in algebraic expressions: S, $[\text{MgATP}]^2$; P, $[\text{Pi}]_{\text{total}}$, and D, $[\text{MgADP}]$. An asterisk (*) and a dagger (†) identify the second and third conformational states, respectively. In step 1a, MgATP binds to the rigorlike AM state to form a collision complex AM$^*$S, which in turn isomerizes in step 1b to form the AM$^*$S state. Crossbridges then detach in step 2 to form the detached (Det) state. The Det state includes all detached states (MS and MDP) and weakly attached states$^{57}$ (AMS and AMDP) in our convention. Hydrolysis occurs in step 3. Because we included several states in the Det state, the kinetic constants within the Det state may influence $k_t$ as deduced in this report. In step 4, the detached crossbridges attach to form the AM$^*$DP state, which is followed by the Pi release from step 5 to form the AM$^*$D state. In step 6, AM$^*$D isomerizes to form AMD, which in turn loses MgADP in step 0 to form the AM state.

The effect of MgATP on exponential process D (Fig 2D) can be explained by a partial scheme of the crossbridge scheme (Fig 7), which includes three states AM, AM$^*$S, AM$^*$S, and transitions between them. Our results are consistent with the hypothesis that step 1a is faster than our speed of observation (135 Hz, 850 s$^{-1}$), and that step 1b is observed by process D. If the binding step is comparable to the speed of observation, this plot becomes linear. The analytical form of the apparent rate constant of the partial crossbridge scheme is as follows:
FIG 5. The apparent rate constants $2\pi b$, $2\pi c$, and $2\pi d$ are plotted in logarithmic scale against the phosphate concentration in panel A. $2\pi b$ (in panel B), $2\pi c$ (in panel C), and $2\pi d$ (in panel D) are also plotted in the linear scale against the phosphate concentration. The MgATP concentration was 5 mM. The data were normalized to the control condition (8 mM phosphate) first, and then averaging was performed over eight experiments and plotted with SEM bars. Data points are connected by straight lines.

\[
2\pi d = \frac{K_{ib}S}{1 + K_{ib}D + K_{ib}S} k_{ib} + k_{-ib}
\]

The derivation of Equation 3 is given in the Appendix (Equation 21A). The data in Fig 2C is fitted to Equation 3 by assuming $K_{ib}D = 0$ (because of the presence of the CP/CK system, the MgADP concentration is less than 20 $\mu$M [35] in our experimental conditions), and the kinetic constants $K_{ib}$, $k_{ib}$, and $k_{-ib}$ are deduced (Table). The best-fit results are entered in Fig 2D with a curved line. As shown in this figure, the results fit adequately to the crossbridge scheme with three states. In myocardial preparations we find that $2\pi c$ is within a factor of three of $2\pi b$ (Fig 2 or 3), and separation is not as good as the order of magnitude separation between $2\pi c$ and $2\pi d$. Since process B is an exponential delay (negative term in Equation 1) and process C is an exponential advance (positive term), we occasionally found that $2\pi b$ and $2\pi c$ came within a factor of two during the data-fitting procedure. This implies that the rate constants of steps 2 and 4 are similar in their order of magnitudes; therefore, it is difficult to separate and independently map them to process C and process B as we did in the rabbit psoas.\[13,16,35\] Consequently, it is more suitable to use the sum $2\pi b + 2\pi c$ and the product $2\pi b \cdot 2\pi c$ to characterize the kinetics involving these two steps than individual apparent rate constants. The sum and the product relate to the kinetic constants by the following equations:

\[
2\pi b + 2\pi c = ak_3 + k_{-3} + k_{-4} + ek_{-4}
\]

\[
2\pi b \cdot 2\pi c = ak_4 k_{-4} + ek_{-4} k_{-4}
\]

FIG 6. Isometric tension (panel A), stiffness ($Y_x$, panel B), and the tension ($T$) to $Y_x$ ratio (panel C) are plotted against the phosphate concentration. The MgATP concentration was 5 mM. The resting tension and stiffness were subtracted, the data were normalized to the control condition (8 mM phosphate), and then averaging was performed over eight experiments and plotted with SEM bars. Tension in the 8 mM phosphate solution averaged $17 \pm 3$ kN/m$^2$, and $Y_x$ averaged $0.80 \pm 0.14$ MN/m$^2$ (mean $\pm$ SEM, n=8).
\[
\begin{array}{cccccccc}
\text{Step 0} & \text{Step 1a} & \text{Step 1b} & \text{Step 2} & \text{Step 3} & \text{Step 4} & \text{Step 5} & \text{Step 6} \\
D & K_{1a} & k_{ib} & k_2 & \text{AMS} \leftrightarrow \text{AMDP} & k_4 & P & k_6 \\
\text{AMD} & \text{AM} & \text{AM'S} \leftrightarrow \text{AM'S} & \downarrow & \downarrow & \Rightarrow \text{AM'DP} & \downarrow & \text{AM'D} \\
K_0 & S & k_{ib} & k_2 & \text{MS} \leftrightarrow \text{MDP} & k_{ib} & K_5 \\
\end{array}
\]

**Fig 7.** Crossbridge scheme consisting of seven crossbridge states and characterized by seven rate constants (k) and three association constants (K) (kinetic constants) that govern transitions between the states. A, actin; M, myosin head; S, MgATP; D, MgADP; P, phosphate; *, second conformational state; †, third conformational state; Det, detached state; X_i, steady-state probabilities of crossbridge states.

Where

\[(6) \quad \alpha = \alpha(S) = K_{1a}K_0S/((1+K_{1b})D + (1+K_{ib})K_2S),\]

with \[0 \leq \alpha < K_{ib}/(1+K_{ib})\]

and

\[(7) \quad \epsilon = \epsilon(P) = K_p/(1+K_p), \] with \[0 \leq \epsilon < 1\]

The derivation of these equations is given in the Appendix (Equations 25A and 26A). Note that Equations 3 through 7 have the following general hyperbolic form:

\[(8) \quad r(v) = [Ev/(1+Kv)] + F\]

where \(r\) is an observed quantity (eg, the sum), \(v\) is the experimental variable (S, D, or P), \(K\) is the association constant, and E and F are linear coefficients. \(K, E,\) and \(F\) are determined by data fitting (least-squares method). Because \(K\) is nonlinear to \(r, K\) must be determined by an iterative procedure. Because E and F are linear to r, standard linear fitting can be applied to find these coefficients. All data fitting was performed according to Equation 8, and \(K, E, F,\) and \(F\) were deduced. These values were then used to calculate individual kinetic constants in Equations 3 through 5.

The sum and the product for the MgATP study are plotted in Fig 8. The data were fitted to Equations 4 and 5 to deduce kinetic constants; the theoretical projection is shown by curved lines in Fig 8. In the actual fitting procedure, the sum \[2\pi + 2\pi c\] and \[2\pi d\] were simultaneously fitted to Equations 3 and 4 to find \(K_{1a}\) (MgATP association constant), which is common to both equations. This procedure was followed by fitting the product to Equation 5 with the already obtained value \(K_0\). As seen in Figs 2C and 8, the fitting is satisfactory, implying that the scheme in Fig 7 is an appropriate representation of the myocardial crossbridge cycle in its response to the MgATP concentration change. The values for \(K_{1a}, k_{ib}, k_{2}, k_{4},\) and \(k_{ib}\) are listed in the Table.

The sum and the product for the Pi study are plotted in Fig 9. The data are fitted to Equations 4 and 5 to deduce kinetic constants, and the theoretical projection is shown by curved lines. In performing the data fitting, \(K_1\) (Pi association constant) of the sum in Equation 4 was determined first by an iterative procedure, and this value was used to perform linear fitting to find the other parameters in Equation 5. As seen in Fig 9, the fit is satisfactory, indicating that the scheme in Fig 7 is also an appropriate representation of the myocardial crossbridge cycle in its response to the Pi concentration change. The result also implies that the Pi binding step is faster than our speed of observation or else one of the

### The Kinetic Constants of Elementary Steps

<table>
<thead>
<tr>
<th>Constant</th>
<th>Unit</th>
<th>Mean ± SEM</th>
<th>n</th>
<th>Best fit</th>
<th>Mean ± SEM</th>
<th>n</th>
<th>Psoas/myocardium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_{1a})</td>
<td>mM⁻¹</td>
<td>0.23 ± 0.04</td>
<td>10</td>
<td>0.82</td>
<td>0.99 ± 0.17</td>
<td>6</td>
<td>0.23</td>
</tr>
<tr>
<td>(k_{ib})</td>
<td>s⁻¹</td>
<td>1880 ± 220</td>
<td>10</td>
<td>263</td>
<td>270 ± 60</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>(k_{-ib})</td>
<td>s⁻¹</td>
<td>1510 ± 110</td>
<td>10</td>
<td>235</td>
<td>280 ± 20</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>(K_{ib})</td>
<td>None</td>
<td>1.29 ± 0.15</td>
<td>10</td>
<td>1.12</td>
<td>0.95 ± 0.18</td>
<td>5</td>
<td>1.2</td>
</tr>
<tr>
<td>(k_{2})</td>
<td>s⁻¹</td>
<td>510 ± 30</td>
<td>10</td>
<td>46.0</td>
<td>48 ± 6</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>(k_{-2})</td>
<td>s⁻¹</td>
<td>132 ± 7</td>
<td>10</td>
<td>12.5</td>
<td>14 ± 2</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>(K_3)</td>
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<td>3.9 ± 0.3</td>
<td>10</td>
<td>3.67</td>
<td>3.5 ± 0.4</td>
<td>6</td>
<td>1.1</td>
</tr>
<tr>
<td>(k_4)</td>
<td>s⁻¹</td>
<td>106 ± 4</td>
<td>11</td>
<td>10.0</td>
<td>11 ± 2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>(k_{-4})</td>
<td>s⁻¹</td>
<td>90 ± 5</td>
<td>11</td>
<td>94</td>
<td>107 ± 15</td>
<td>8</td>
<td>0.8</td>
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<tr>
<td>(K_4)</td>
<td>None</td>
<td>1.20 ± 0.07</td>
<td>11</td>
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<td>0.11 ± 0.02</td>
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<td>11</td>
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<tr>
<td>(K_3)</td>
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<td>0.19 ± 0.02</td>
<td>11</td>
<td>0.0568</td>
<td>0.060 ± 0.011</td>
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<tr>
<td>(K_{1a}/K_4)</td>
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<td>0.16</td>
<td>0.54</td>
<td>0.55</td>
<td>0.3</td>
<td></td>
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</tr>
<tr>
<td>(K_{1a}/K_4)</td>
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<td>1.45</td>
<td>1.53</td>
<td>1.82</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data from individual experiments were fitted to respective equations first, and then averaging was performed on the fitted parameters. The best-fit parameters were obtained by fitting all the available data to respective equations. The fraction is the result of the division of the averaged rabbit psoas value by the averaged ferret myocardium value.
plots in Figs 5B through 5D would become linear. The values for $k_1$, $k_2$, and $K_s$ are included in the Table.

Comparison of $Y_s$ of Control Activation With Rigor Stiffness

To determine the fraction of attached crossbridges directly, the myocardial preparation was activated with the control activating solution (5 mM MgATP, and 8 mM Pi), and the complex modulus $Y(f)$ was obtained as described. From $Y(f)$, $Y_s$ was deduced according to Equations 1 and 2. The preparation was then brought into high-rigor condition by three full volume changes with the rigor solution, and the dynamic modulus at 100 Hz ($Y_{100}$) was determined. The choice of frequency in determining rigor stiffness is immaterial, because stiffness in a rigor preparation is almost independent of frequency. The resting modulus was subtracted in the usual way. We then calculated the fraction of attached crossbridges during control activation by $Y_s/Y_{100}$, and this value was averaged. The averaged fraction was 51±4% (mean±SEM, n=18). Thus, it can be concluded that 51% of the available crossbridges were attached and 49% were detached during control activation.

Discussion

MgATP Binding and ATP-Isomerization Steps

We studied the effect of MgATP and Pi on the apparent rate constants. We found that the apparent rate constants $2\pi b$, $2\pi c$, and $2\pi d$ increased with an increase in the MgATP concentration in the low millimolar range and were saturated in the high millimolar range (Fig 2). Of these apparent rate constants, $2\pi d$ is about 10 times $2\pi c$, and these values are adequately separated. This justifies isolating steps 1a and 1b in the scheme in Fig 7 from the rest of the crossbridge cycle. In
other words, for frequencies that characterize process D, slower reactions can be assumed not to occur. From the MgATP dependence of $2\pi d$, we were able to deduce the kinetic constants $k_{1a}$, $k_{1b}$, and $k_{e-1b}$ (Table). For comparative purposes, the kinetic constants deduced from rabbit psoas\textsuperscript{16} are also included in the Table. We find $K_{d}$ to be 0.99 mM\textsuperscript{-1}, which compares to 0.23 mM\textsuperscript{-1} in rabbit psoas under the same activating conditions. Thus, it can be concluded that the substrate (MgATP) binds four times more strongly to crossbridges in myocardium than in psoas. Strong binding may occur because the MgATP molecule may fit better to the nucleotide binding site on the myosin head in myocardium than in psoas. The kinetic constants $k_{1b}$ and $k_{e-1b}$ are found to be 270 s\textsuperscript{-1} and 280 s\textsuperscript{-1}, respectively, and these values compare with 1880 s\textsuperscript{-1} and 1510 s\textsuperscript{-1}, respectively, in psoas. Thus, it can be concluded that the ATP isomerization and its reversal steps are six to seven times slower in myocardium. Interestingly, the equilibrium constant $K_{d}$ does not vary between myocardium and psoas (Table). This indicates that the free energy change associated with the ATP isomerization while bound to the myosin head is the same in myocardium and psoas.

Although the effect of MgATP on $2\pi d$ is significant (Figs 2A and 2D), the effect of Pi on this apparent rate constant is very small or absent (Figs 5A and 5D). This observation is consistent with the scheme in Fig 7. Because steps 2 and 4 are much slower than steps 1a and 1b, the Pi binding in step 5 is isolated from step 1b. Consequently, $2\pi d$ is not influenced by the Pi concentration. The same argument holds true for step 6. If step 6 is faster, then it follows that an increase in the Pi concentration would diminish $2\pi d$. On the basis of the fact that $2\pi d$ does not decrease with an increase in the Pi concentration (Figs 5A and 5D), we conclude that step 6 is much slower than step 2 or 4.

Crossbridge Detachment Step

The difference of the apparent rate constants $2\pi b$ and $2\pi c$ is not large, and $2\pi c$ is within a factor of three of $2\pi b$ (Figs 2 and 5). This implies that the rate constants of elementary steps 2 and 4 are similar, and they are difficult to study independently, as in the case of rabbit psoas fibers\textsuperscript{13,35} Under such circumstances, it is more suitable to use the sum $2\pi b+2\pi c$ and the product $2\pi b \cdot 2\pi c$ of these rate constants to characterize the kinetics involving steps 2 and 4. Furthermore, Equations 4 and 5 demonstrate that the relation between observed quantities and the kinetic constants of the elementary steps is more readily determined with the sum and the product than with the apparent rate constants themselves. In fact, both the sum (Equation 4) and the product (Equation 5) relate to the MgATP or Pi concentration by the simple hyperbolic relation represented by Equation 8. The good fit of the MgATP effect (Figs 2D and 8) and the Pi effect (Fig 9) implies that the crossbridge scheme is a logical representation of our data. Our data are consistent with the hypothesis that $2\pi c$ is mostly influenced by step 2 and to a lesser extent by step 4. Thus, the major step that influences process C in ferret myocardium is the crossbridge detachment step, as it is in rabbit psoas.\textsuperscript{12,35,43}

We found that the rate constants of the crossbridge detachment step $k_{1}$ and $k_{-1}$ are 48 s\textsuperscript{-1} and 14 s\textsuperscript{-1}, respectively, and these compare with 510 s\textsuperscript{-1} and 132 s\textsuperscript{-1}, respectively, of rabbit psoas (Table). Thus, we conclude that the crossbridge detachment step is about 10 times slower in myocardium than in psoas. The equilibrium constant $K_{d}$ varies little between myocardium and psoas, implying that the free energy change associated with step 2 is similar in the two preparations. In solution studies, the second-order rate constant of MgATP binding and subsequent reaction was reported to be 2500 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} in rabbit white muscle myosin and 500 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} in cardiac myosin\textsuperscript{32}; hence, their ratio is 5. In the structured muscle system, the composite second-order rate constant $K_{\text{composite}}$ is 430 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} in psoas, which compares to 270 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} in myocardium; hence, their ratio is 1.6. Similarly, the composite second-order rate constant $K_{\text{composite}}$ is 150 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} in psoas, which compares to 45 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} in myocardium; hence, their ratio is 3.4. Although a direct comparison of these numbers in solution studies of proteins and in muscle fiber studies may not be appropriate because of the vast difference in conditions, the comparison of the ratio may provide useful information; the ratios may reflect a true difference between the fast-twitch skeletal muscle and the cardiac muscle preparations.

Crossbridge Attachment and Phosphate Release Steps

From the Pi study, we found $k_{1}$ to be 11 s\textsuperscript{-1} in myocardium, which compares to 106 s\textsuperscript{-1} in psoas (Table). Therefore, it can be concluded that the crossbridge attachment step is 10 times slower in myocardium than psoas. In solution studies, the association of actin and the myosin product is reported to be 80 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} for cardiac myosin and 520 mM\textsuperscript{-1} \cdot s\textsuperscript{-1} for chicken posterior latissimus dorsi myosin\textsuperscript{32}; hence, their ratio is 6.5. This ratio varies little from the ratio on muscle fibers of the current study. It should be mentioned here that the association of myosin product with actin in solution results in the formation of the weakly attached AMDP state, whereas the crossbridge attachment step in fibers results in the formation of the strongly attached AM*DP state. Thus, a direct comparison of these steps may appear inappropriate. It must be emphasized, however, that our measured $k_{1}$ is presumably a composite rate constant that includes the preceding step(s); therefore, the comparison may not be unreasonable.

Our data are consistent with the hypothesis that $2\pi b$ is influenced mostly by step 4 and to a lesser extent by step 2. This interpretation is consistent with the experimental observation by Steiger et al\textsuperscript{36} on rabbit papillary muscles that stiffness increased during the delayed rise in tension following a step-length increase; this phase corresponds to process B. The interpretation is also consistent with our own conclusion on rabbit psoas\textsuperscript{13,16} that process B correlates with crossbridge attachment step 4.

Another interesting observation is that the rate constant of the reverse attachment step ($k_{-a}$) is 107 s\textsuperscript{-1} in myocardium, which is not very different from 90 s\textsuperscript{-1} in psoas (Table). This suggests that the reversal of the attachment step may occur irrespective of the myosin type, and possibly spontaneously. The equilibrium constant of the attachment step ($K_{a}$) is 0.11 in myocardium, compared with 1.2 in psoas (Table); this implies that the free energy change of the attachment step is different between psoas and myocardium by RTln(1.2/0.11) (5.8
This difference suggests that the structure of the myosin head in the AM*DP state is somewhat different between psoas and myocardium. Energetically, this difference is compensated for by increased $K_{m}$ and decreased $K_{d}$ (see below), and $K_{m}K_{d}/K_{d}$ is approximately the same between rabbit psoas and ferret myocardium (Table).

We found that the Pi association constant ($K_{r}$) is 0.06 mM$^{-1}$ in myocardium, which compares to 0.19 mM$^{-1}$ in psoas (Table). Thus, we conclude that Pi binds to myocardial crossbridges three times more weakly than to psoas. In the solution studies, the Pi association constant is 0.1 M$^{-1}$ to 0.01 M$^{-1}$; hence, the Pi release step is practically irreversible, and the large free energy liberated is lost as heat. In muscle fibers, the free energy released by hydrolysis is presumably stored as potential energy in the elastic portion of the crossbridges (and in structures in series with crossbridges), which is sensed as force; hence, the Pi release step is reversible ($K_{r}P=1$), as originally observed by Ruegg et al$^{53}$ on insect flight muscles.

Rate-Limiting Step

Step 6 is the slowest forward reaction in the crossbridge cycle; ie, step 6 is the rate-limiting step. If it is a faster reaction, we could not explain the MgATP, MgADP, and Pi effects on three apparent rate constants. For example, if the slowest step is in step 3, then it follows that Pi will decrease 2$nb$, which is contrary to our results (Fig 5D). It also follows that MgATP will either decrease 2$nb$ or will not affect 2$nb$ at all, which is also contrary to our results (Fig 2B). Our conclusion that step 6 is the rate-limiting step in myocardium is the same as that in rabbit psoas fibers$^{13,16}$ and is consistent with the hypotheses of others.$^{60}$

Uniqueness of the Crossbridge Scheme

It is important to emphasize that the scheme in Fig 7, which was developed on the basis of skinned fiber experiments, is unique to describe our data. After an exhaustive search to fit the available data to various crossbridge schemes, we concluded that no other scheme with the same degree of simplicity fits our data. For instance, if we correlate step 1b to process C and step 2 to process D, then 2$md$ would become insensitive to the MgATP concentration because a slow step 1b isolates step 2 from the MgATP binding step 1a. This does not coincide with our results (Fig 2D). The crossbridge scheme based on MgATP and Pi effects on ferret myocardium turned out to be exactly the same as that derived from rabbit psoas fibers.$^{16,53}$ The partial scheme that includes three states surrounding the Pi-release step (Det, AM*DP, and AM*D) is consistent with the conclusion based on caged Pi experiments$^{10,14}$ and the conclusion based on pressure-release experiments$^{11}$ on rabbit psoas. It is also important to point out that the scheme, which was developed on the basis of skinned muscle preparations, is close to that developed from solution studies on isolated and reconstituted muscle proteins using biochemical techniques$^{5,6,8}$ except that we identified fewer states. It is possible that additional parallel hydrolysis pathways, such as predicted by solution studies, may exist. However, these additional pathways must play a minor role compared with the pathway shown in the crossbridge scheme, which plays the pre-dominant role in the transduction mechanism in skinned fibers.

Probability of Crossbridge States and Stiffness

Whereas the apparent rate constants are indicators of transitions between crossbridge states, isometric tension and stiffness ($Y_{e}$) are indicators of the number of crossbridges in attached states. For this reason, the probability of crossbridges in each state was calculated on the basis of Equations 9A through 15A (Appendix) with kinetic constants listed in the Table and plotted as functions of MgATP concentration (Fig 10A) and Pi concentration (Fig 10B). In the control activating condition (5 mM MgATP$^{2-}$, 8 mM Pi) in ferret myocardium, the distribution was as follows: AM, 3%; AM$^{1+}$, 15%; AM*PS, 14%; Det, 50%; AM*DP, 6%; and AM*D, 12%. In contrast, the distribution in rabbit psoas was as follows$^{54}$: AM, 5%; AM$^{1+}$, 0%; AM*PS, 7%; Det, 28%; AM*DP, 33%; and AM*D, 22%. Thus, it can be concluded that in myocardium crossbridges are more populated in the AM*PS, AM*PS, and Det states, and less populated in the AM*DP and AM*D states than in rabbit psoas. Fig 10 also plots the sum of probabilities of attached crossbridges in myocardium (labeled $A_{det}$ in the figure). In the control activating condition, the calculated sum of the attached crossbridges was 50%. This number agrees extremely well with the direct measurement (51±4%) of the attached crossbridges determined from $Y_{e}$ during the control activation and $Y_{100}$ (stiffness at 100 Hz) during rigor. This fact demonstrates the suitability of our scheme and the accuracy of our measurements.

The decrease in $Y_{e}$ with an increase in the MgATP (Fig 3B) or Pi (Fig 6B) concentration is consistent with Fig 10 in that the attached crossbridge numbers decrease with an increase in the MgATP or Pi concentration. This is because $Y_{e}$ is the elastic modulus of the muscle detected by the fast length change; hence, $Y_{e}$ is proportional to the number of attached crossbridges. In Fig 10A, the magnitude of change of the numbers of attached crossbridges appears to be larger than the corresponding change in $Y_{e}$ (Fig 3B). However, this is not the case, because the theoretical calculation (Fig 10A) includes the 0 mM MgATP point, whereas the lowest MgATP concentration used in experiments (Fig 3B) is 0.1 mM. In fact, at 0.1 mM MgATP, the attached crossbridge probability reduces to 80%; hence, the two plots become similar. The same holds true for the Pi effect. Experimentally, the 0 mM Pi condition is impossible to achieve because of the continuous liberation of Pi due to hydrolysis of ATP$^{13,25,50}$ and the contaminating Pi in the activating solution (mostly associated with CP). In addition, since step 6 is finite (instead of 0), some crossbridges are distributed in the states from AM through AM*DP (X$_{m}$ through X$_{c}$, as defined in Fig 7) in the absence of Pi. These considerations effectively displace the absissa of Fig 10B to the right when compared with the experimental results. With a displacement of 1.3 mM, the probability of attached crossbridges in the absence of exogenous Pi becomes 70%, and an exact match with the experimental data of Fig 6B and the theoretical projection (Fig 10B) occurs. In rabbit psoas, we found the corresponding value to be 0.6 mM; this value may be larger in myocardium because of the larger diameter.
Isometric Tension

It is interesting to observe that the ratio of tension to $Y_e$ slightly increases with an increase in the MgATP concentration (Fig 3C), and the ratio slightly decreases with an increase in the Pi concentration (Fig 6C). As seen from the SEM in both figures, the effect is significant. Since $Y_e$ is proportionate to the number of attached crossbridges, we assume that the ratio of tension to $Y_e$ represents average tension per crossbridge. From these observations, we infer that an increase in the MgATP concentration shifts the crossbridge population from a lower-force state to a higher-force state, whereas an increase in the Pi concentration shifts the crossbridge populations from a higher-force state to a lower-force state. In the crossbridge scheme, it must be evident that MgATP shifts the equilibrium to the right, whereas Pi shifts the equilibrium to the left. If we assume that force/crossbridge\textsuperscript{56} in the AM state is lower than that in the AM* D state, we can explain the observed effects of MgATP and Pi on the ratio of tension to $Y_e$. In fact, this hypothesis is consistent with our observation that the AM* D state in rabbit psosas supports 26% more tension compared with the AM state.\textsuperscript{56}

Tension at 16 mM Pi in ferret myocardium was 54% of that at 0 mM Pi in our observations (Fig 6A) and lower than the corresponding value in rabbit psosas (60%)\textsuperscript{13} under the same activating conditions. Gold and Nosek\textsuperscript{3} observed an even larger difference between rabbit myocardium (45%) and psosas (69%) at 20 mM Pi. Similarly, Kentish\textsuperscript{65,55} observed 30% to 34% remaining tension at 20 mM Pi in rat myocardium. Although it might appear that these observations are contradictory to the above conclusion that Pi binding is weaker in myocardium than psosas, this is not the case as the following calculation demonstrates. Based on Equation 15A and the kinetic constants given in the Table, the number of attached crossbridges is calculated to be 47% in myocardium and 69% in psosas at 16 mM Pi and 5 mM MgATP. The attached crossbridge number in myocardium is smaller primarily because of lower $K_i$ in myocardium than in psosas. This difference in attached crossbridge numbers qualitatively explains the larger Pi effect on isometric tension in myocardium than in psosas. This argument assumes both AM* D and AM* D to be the tension states as in the case of rabbit psosas.\textsuperscript{13} This assumption is consistent with the observation that at 16 mM Pi there is a significant amount of tension (54%, Fig 6A), whereas the probability of the AM* D state is 7% to 12% (Fig 10B) and too small to account for tension. Therefore, we conclude that the AM* D state also supports tension in addition to the AM* D state; hence, step 4 corresponds to the force generation step in myocardium as in rabbit psosas.\textsuperscript{13} There are additional factors that determine isometric tension, including tension per crossbridge state\textsuperscript{56} as well as the definition of the state when no Pi is added exogenously. These subjects are beyond the scope of the present study, and the quantitative analysis must come from further studies.

Possible Artifacts in Measurements

In cardiac preparations, there are more parallel elasticity and end compliance than in rabbit psosas. Of these, the parallel elasticity is not a concern, because it is included in the parameter $H$ (Equation 1) and it does not affect crossbridge kinetics represented by processes B, C, D and as measured by a small-length perturbation method. The end compliance may be a concern, and its significance must be deduced by an experiment in which the segment or sarcomere length is controlled. In the absence of such control, the significance of the end compliance can be estimated by the tension to stiffness ratio. This quantity represents the degree of instantaneous length release required to abolish full active tension, and it becomes larger with end compliance.
With our experiments, the ratio was found to be 1.85% in skinned ferret myocardium in an activating solution that approximated the intracellular composition of myocytes. In comparison, Pollack and Krueger estimated the ratio to be 1.6% by measuring sarcomere length changes in the midsection of rat myocardium; Shibata et al. observed 1.8% by a segment-clamp experiment in rabbit papillary muscles. Our value is not very different from these length-controlled measurements, indicating that the effect of end compliance should be small in our preparations. An additional concern may be that the amplitude of the sinusoidal length change may not be the same at a different force level, owing again to possible end compliance. However, this should not cause a problem in our analysis method, because the complex modulus $Y(f)$ is the ratio of stress to strain, and for the first approximation, a small variation in amplitude does not affect the modulus measurement. Therefore, we conclude that the end compliance, if such existed, does not underlie our deduction of the crossbridge scheme or the kinetic constants of elementary steps.

**Implication of the Current Research**

The results of the current research on myocardium may be significant in exploring the mechanisms of cardiac exhaustion and dysfunction. After ischemia or hypoxia, the heart muscle fails to contract and undergoes an increase in diastolic tension. However, the cellular basis of how myocardial function is altered by these conditions has been difficult to investigate. This is in part because the mechanisms underlying cardiac failure are complex because of the simultaneous changes in myoplasmic ionic constituents that modify and regulate crossbridge functions. The present study with skinned preparations will give insights into the specific role of MgATP and Pi in the elementary steps of the crossbridge cycle. Our finding that cardiac muscle is more resistant to ATP depletion and Pi buildup than skeletal muscle is intriguing and important in understanding the durability of myocardium. Thus, our approach is complementary to recently developed techniques to measure intracellular concentrations of crucial ions such as Ca$^{2+}$, H$^+$, MgATP, MgADP, and Pi that regulate myocardial contractility. It is expected that these techniques will play significant roles in enriching our knowledge of the mechanisms and consequences of cardiac dysfunctions.

**Appendix**

**Steady-state Solution of Crossbridge Scheme**

In the following analysis, $X_i$ $(X_0, X_1, \ldots, X_4)$ represents the steady-state probability of crossbridges in the respective state as shown in Fig 7. The steady-state probability can be obtained by assuming the mass action law (equilibrium) as an approximation for steps 0 through 5. The mass action law cannot be applied to the rate-limiting step 6.

(1A) $X_0 = X_0 K_D$

(2A) $X_1 = X_0 K_p S$

(3A) $X_2 = X_1 K_p$

(4A) $X_3 = X_1 K_2$

(5A) $X_4 = X_2 K_4$

(6A) $X_5 = X_4 K_2$

In addition, the values of $X_i$ are probabilities (conservation rule): $X_0 + X_1 + X_2 + X_3 + X_4 + X_5 = 1$

Thus, from Equations 1A through 7A, we obtain

(8A) $X_0 = X_0 K_D D K_P / M$ (AMD state)

(9A) $X_1 = X_1 K_P / M$

(10A) $X_2 = X_1 K_P S / M$ (AM1S)

(11A) $X_3 = X_1 K_P S K_P / M$ (AM’S)

(12A) $X_4 = X_1 K_D K_4 / M$ (Det)

(13A) $X_5 = X_1 K_D K_4 S / M$ (AM*DP)

(14A) $X_6 = X_1 K_D K_4 S / M$ (AM*D)

(15A) $X_{2i} = X_0 + X_1 + X_2 + X_3 + X_4$

$= 1 - X_5 + 1 = 1 - K_p S K_p K_p / M$

where $M = K_D S K_D + K_P [1 + K_D D + K_P S (1 + X_1 + X_2 + X_3)]$.

It is apparent from Equations 8A through 14A that they have the general hyperbolic form of Equation 8 when plotted against S, D, or P, and as shown in Fig 10.

**Transient Solution of Step 1b**

To delineate the deviation from the steady-state probability $X_i$, we use the lower case $x_i(t)$ for the time (t)-dependent transient solution. Under the conditions of these experiments, the complete crossbridge cycle operates at a steady-state rate defined by the slowest step in the overall scheme. The minute perturbations of the sinusoidal oscillation (0.25% peak to peak) then permit the faster reactions to be studied as uncoupled pieces of the total scheme. When we focus on the frequencies that characterize step 1b (process D), steps 0 and 1a are faster; hence, these steps can be approximated by the mass action law, and Equations 1A and 2A can be directly applied to $x_i$. Evidently, Equation 3A is not applicable. Steps 2, 4, and 6 are much slower than step 1b; hence, they effectively do not occur for frequencies that characterize process D; i.e., these steps are uncoupled from process D. This procedure isolates steps 2 through 6 from step 1b. Thus, the master equation that characterizes step 1b is as follows:

(16A) $\dot{x}_0 + x_1 + x_2 = -k_1 x_0 x_0 + k_{-1} x_2 = -x_2$

where a dot above $x_i$ indicates differentiation with respect to the time $t$ (a dot above $x_i$ indicates differentiation with respect to the time $\dot{x}_i = dx_i / dt$). The conservation rule is as follows:

(17A) $x_0 + x_1 + x_2 + x_3 = 0$

After substituting Equations 1A and 2A into Equation 16A, we obtain

(18A) $\dot{x}_2 / \beta = -k_1 x_0 x_0 + k_{-1} x_2 = -x_2$

where $\beta = K_p S (1 + K_D D + K_p S)$.

If we similarly substitute Equations 1A and 2A into Equation 17A, we obtain

(19A) $x_0 + x_1 = 0$

If we eliminate $x_1$ from Equations 18A and 19A, we obtain

(20A) $\dot{x}_0 = -k_1 x_0 x_0 + k_{-1} x_0$
The apparent rate constant of Equation 20A is then given by

\[ 2\pi d = \beta k_{1b} + k_{-1b} \]

By fitting the MgATP effect to Equation 21A, we can determine \( K_{1a}, k_{1b}, \) and \( k_{-1b}. \)

**Transient Solution of Steps 2 and 4**

When we focus on the frequencies that characterize steps 2 and 4 (processes B and C), steps 0, 1a, 1b, and 5 are faster. Thus, these steps can be approximated by the mass action law, and Equations 1A through 3A and 6A can be directly applied to \( x_t. \) Evidently, Equations 4A and 5A are not applicable. Step 6 is slower than steps 2 and 4, so we assume that this step effectively does not occur for frequencies that characterize processes B and C. Therefore, the master equations that characterize steps 2 and 4 are

\[
\begin{align*}
\dot{x}_0 + \dot{x}_1 + \dot{x}_2 + \dot{x}_3 &= -k_{1a} x_1 + k_{x_3} x_4 \\
\dot{x}_{xa} &= k_{x_3a} (x - 2 + k_{2a}) x_3 + k_{4a} x_3 \\
\dot{x}_4 &= -k_{x_3a} x_4 - k_{-1a} x_3
\end{align*}
\]

After substituting Equations 1A through 3A and Equation 6A into Equation 22A, we obtain

\[
\frac{d}{dt} \frac{d}{dt} x_4 = \frac{a k_{1a}}{1 + k_{2a} x_3 + k_{3a} x_4 + k_{4a} x_3}
\]

where \( a \) and \( e \) are defined in Equations 6 and 7. Note the similarity of Equations 20A and 23A. The apparent rate constants are given by the eigenvalues of the 3×3 matrix in Equation 23A, and they are the roots of the following cubic equation:

\[
r^3 - (a k_{1a} + k_{2a} x_3 + k_{3a} x_4 + k_{4a} x_3) r^2 + (a k_{1a} k_{2a} x_3 + a k_{1a} k_{3a} x_4 + a k_{1a} k_{4a} x_3 + k_{2a} k_{3a} x_4 + k_{2a} k_{4a} x_3 + k_{3a} k_{4a} x_3) r + k_{2a} k_{3a} k_{4a} x_3 = 0
\]

Therefore, based on the roots and the coefficients' relationship of Equation 24A

\[
2 b + 2 \pi c = a k_{1a} + k_{2a} x_3 + k_{3a} x_4 + k_{4a} x_3
\]

for the two roots \( 2 b \) and \( 2 c. \) Note that another root of Equation 24A is 0, and this provides the constant term. Equations 25A and 26A are the same as Equations 4 and 5. By fitting the MgATP and Pi effects to Equations 25A and 26A, we can determine \( K_{xa}, k_{1a}, k_{2a}, k_{3a}, k_{4a}, \) and \( K_r. \)

If \( 2 b < 2 \pi c \) and \( a k_{1a} + k_{2a} x_3 + k_{3a} x_4 + k_{4a} x_3, \) then Equations 25A and 26A reduce to

\[
2 \pi c = a k_{1a} + k_{2a}
\]

\[
2 \pi b = a k_{1a} + k_{4a}
\]

where

\[
2 \pi c = a k_{1a} + k_{4a}
\]

\[
\sigma a k_{1a} (1 + k_{2a})
\]

\[
= K_{xa} K_{x_3} [1 + K_{2a} D + (1 + K_{2a} + K_{1a} K_{2a}) K_{x_3} S]
\]

Equations 27A, 28A, and 29A are identical to Equations 35, 41, and 6, respectively, of our earlier report on rabbit psoas.16

In cardiac muscles, the apparent rate constants \( 2 b \) and \( 2 c \) are within a factor of three; hence, \( a k_{1a} + k_{2a} x_3 + k_{3a} x_4 + k_{4a} x_3 \) is not a good approximation. Therefore, Equations 25A and 26A must be used to deduce the rate constants of elementary steps instead of Equations 27A and 28A.

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