MYSTERIES ABOUT AMPLITUDE AND EFFICIENCY OF CROSS-BRIDGE POWERSTROKE

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1. THE HUXLEY CONTRACTION MODEL

Since the monumental discovery of the sliding filament mechanism in the hexagonal lattice of thick and thin filaments by H. E. Huxley and his colleagues (Huxley and Hanson, 1954), the contraction model of A. F. Huxley (1957) has long been central in the field of muscle mechanics. The main framework of this model is as follows:

1. Muscle contraction results from cyclic attachment and detachment between the myosin heads, i.e. the cross-bridges, and the sites on the thin filament. A cross-bridge splits one ATP molecule in each attachment-detachment cycle.

2. As shown in Fig. 1A, a cross-bridge (M), connected to the thick filament with springs (S_1 and S_2), is fluctuating around its equilibrium position (0) due to thermal agitation. When it attaches to an active site (A) on the thin filament. it exerts either positive or negative force (F) proportional to its distance (x) from 0 by pulling S_1 or S_2 ($F = 0$ when $x = 0$, and $F = kx$ when $x > 0$ or $x < 0$, k is the stiffness of the spring.

3. The rate constants for attachment of M to, and its detachment from, A (f and g, respectively) are functions of x as shown in Fig. 1B. M attaches to A if M and A are within the range of x where f has finite values ($0 \leq x \leq h$) to exert positive force. If the A-M link moves across 0 to the negative x region, it breaks fairly rapidly because f is zero and g has a large value.

In Fig. 1C, the steady-state distribution of A-M links (expressed by fractional numbers $0 \leq n \leq 1$) is shown against x at various steady shortening velocities V (expressed relative to V_{max}). In the isometric condition ($P = P_0$, $V = 0$), A-M links show square-shaped distribution only in the positive x region where f has finite values ($0 \leq x \leq h$, Fig. $1C_1$). As the steady shortening velocity increases, the number of A-M links

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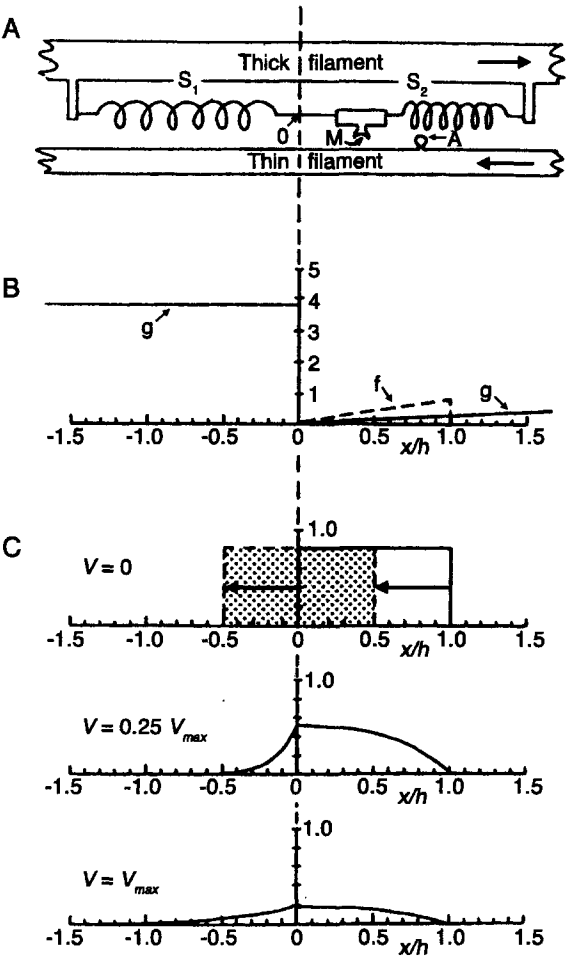

Figure 1. A. F. Huxley's contraction model (Huxley 1957). (A) Diagram showing the myosin head M connected to the thick filament with springs S_1 and S_2, and the active site A on the thin filament. Direction of relative myofilament sliding is indicated by arrows. (B) Dependence of f and g on the distance x of M from the equilibrium position 0. M can only attach to A to form an A-M link at $0 \leq x \leq h$, where f has a finite value. (C_1 – C_3) Diagram showing distribution of A-M links with respect to x at three different steady-shortening velocities: in C_1 the broken line and dotted area show a rapid shift of A-M link distribution in response to a quick length decrease of an isometrically tetanized muscle fiver to drop the force to zero; C_2 and C_3 show changes in A-M link distribution with increasing steady velocity of shortening.

exerting positive forces decreases as a result of a moderate value of f; the proportion of M that slides past A without formation of A-M link increases as the velocity of filament sliding increases. On the other hand, A-M links brought into the negative x region tend to exist over a fairly large distance as the sliding velocity increases (Fig. $1C_2$). Finally, the sliding velocity reaches a value, under which the positive and negative forces exerted by A-M links at both sides of 0 are equal (Fig. $1C_3$). This velocity corresponds to the maximum shortening velocity (V_{max}) under zero external load.

In this model, the myofilaments are assumed to be rigid, and the series elastic component SEC therefore originates only from S_1 and S_2 connecting M to the thick filament. During the plateau of isometric tetanus, all A-M links generate positive forces ≥ 0 by pulling S_1. The quick decrease in force in response to quick release occurs due to elastic recoil of stretched S_1, resulting in a rapid shift of the A-M link distribution into the negative x region. If the shift amounts to h / 2, the positive and negative forces on S_1 and S_2 become equal immediately after the release, and the force just drops from P_0 to zero (dotted area in Fig. $1C_1$). Since the amount of quick release required to drop P_0 to zero is about 1 % of muscle length (i.e. about 10 nm in each 1 _ m long half-sarcomere), the value of h is ~ 20 nm.

The Huxley contraction model can well explain the force-velocity and the force-energy relations in skeletal muscle (A. V. Hill, 1938). Its simplicity and beauty have attracted attention of investigators in the field of muscle mechanics, and a number of experiments have hitherto been made and the results obtained have been interpreted within the framework of this model. In this article, we aim at considering some selected topics in the field of muscle mechanics, which still remain mysterious and should be clarified in future.

2. TRANSIENT PHENOMENA FOLLOWING STEP CHANGES IN LOAD AND IN LENGTH

An effective means to obtain insight into the mechanism producing force and motion in muscle is to apply step changes in load to an isometrically contracting muscle fiber to examine the resulting isotonic shortening. Using this technique, Civan and Podolsky (1966) found that, following step decreases in load from P_0 to $P < P_0$, the fiber showed oscillatory changes in shortening velocity, i.e. isotonic velocity transients, before a steady isotonic shortening determined by the new load P was reached (Fig. 2A). The isotonic condition is suitable for simulating the transient phenomena by determining the A-M link distribution for each consecutive time segment and then shift the A-M link distribution along the force (x) axis to maintain the isotonic condition. In the Huxley contraction model, the square-shaped A-M link distribution in the isometric condition shifts to the left immediately after a step load decrease. During the subsequent time segment, the A-M links increase in the positive x region, while they decrease in the negative x region. To maintain the isotonic condition, therefore, the new A-M link distribution should be shifted to the left. The above process is repeated until the steady A-M link distribution associated with steady isotonic shortening is reached.

Podolsky and his colleagues (Podolsky, Nolan and Zavaler, 1969) showed that the oscillatory isotonic velocity transients could only be simulated by the rate constants and the cross-bridge force as functions of x as shown in Fig. 2C. The Podolsky model differs from the Huxley contraction model in that (1) the rate function for making A-M links f is large so that every M passing through the positive x region forms A-M link, (2) the rate constant for breaking A-M link g in the negative x region is very small near the equilibrium position 0, and suddenly increases for x larger than a critical value. They also made additional assumptions that (1) the series elasticity originates not only from the A-M link but also from the thin filament in the I-band, and (2) breaking of A-M links are associated with ATP splitting only in the small g region, while A-M links brought into the large g region are broken by being pushed by other A-M links exerting positive

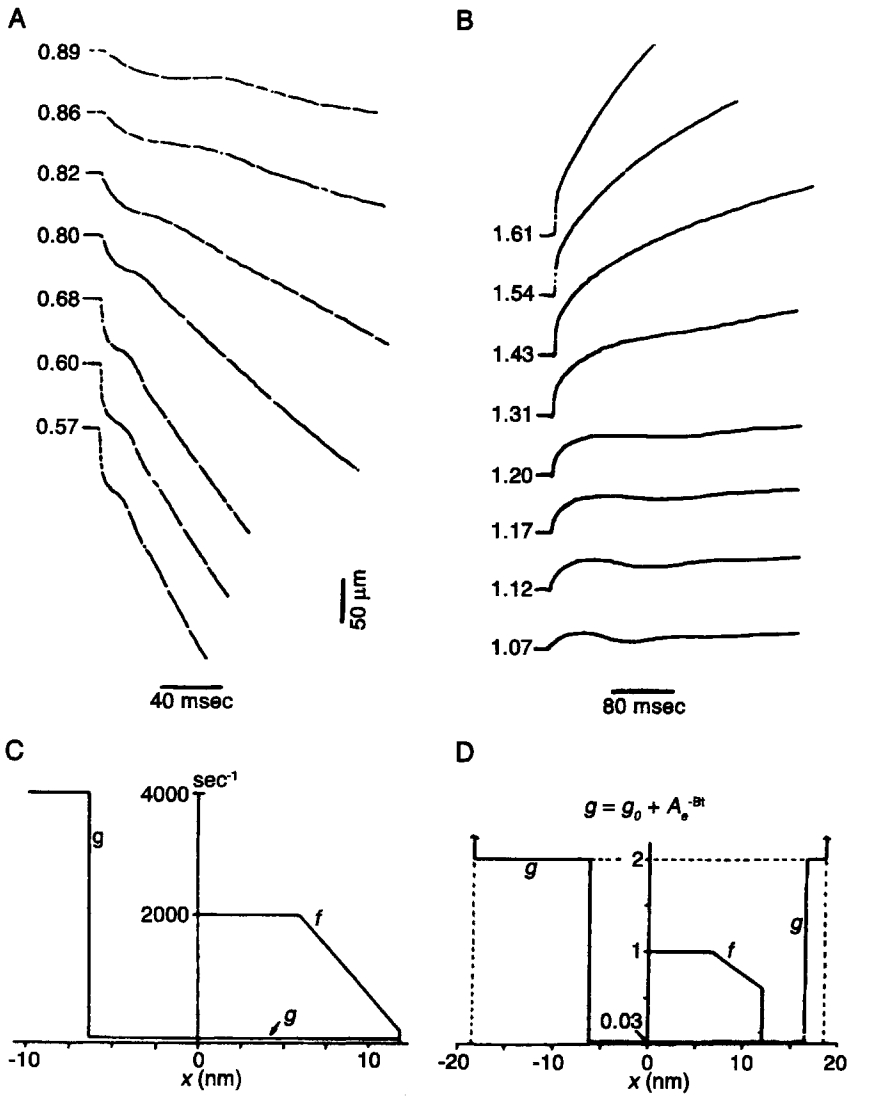

Figure 2. Isotonic velocity transients and the contraction models to stimulate the transients. (A) Isotonic velocity transients following quick decreases in force (= load) from P_0 to $P < P_0$. Note oscillatory changes in shortening velocity before steady velocities are reached. (B) Isotonic velocity transients following quick increases in force from P_0 to P . P_0. Note oscillatory changes in fiber length in response to small increases in force. In both A and B, the values of P relative to P_0 are shown alongside each record (Sugi and Tsuchiya 1981). (C) Podolsky-Nolan model for calculating the velocity transients following quick decreases in force. Note large values of f and a small value of g between f and the major part of g in the negative x region (Podolsky and Nolan 1973). (D) Sugi-Tsuchiya model for calculating isotonic velocity transients following both quick decreases and quick increases in force. Note an additional gap between f and the major part of g in the positive x region together with the assumption that a quick increase in force produces an instantaneous increase in g followed by exponential recovery at every value of x (Sugi and Tsuchiya 1981).

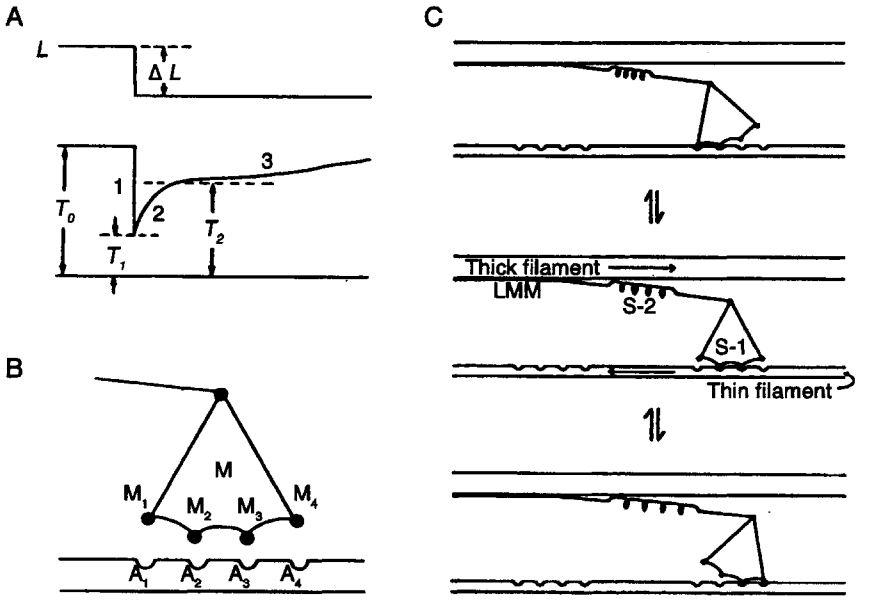

Figure 3. Huxlley-Simmons contraction model (A. F. Huxley and Simmons 1971). (A) Diagram shoing the components of the isometric force transients following a quick release. (B) Diagram showing the myosin S-1 head (M) with four binding sites (M_1-M_4) and part of the thin filament with four binding sitses (A_1-A_4). (C) Mechanism of force generation by rotation of the myosin head on the thin filament by pulling the spring (S-2) between the myosin head and the thick filament (A.F. Huxley 1974).

force. The Podolsky model described above can also account for the force-velocity and the force-energy relations in skeletal muscle, when some additional assumptions are made.

Later, Sugi and Tsuchiya (Sugi and Tsutiya, 1981) studied the isotonic velocity transients following step increases in load from P_0 to $P > P_0$ with the results shown in Fig. 2B. The most characteristic feature was that the transients following a small load increase showed a remarkable length oscillation consisting of alternate lengthening and shortening of the fiber. They could simulate the results using the contraction model show in Fig. 3D with the additional assumption that the value of g increases everywhere immediately after a step load increase and then returns to the initial value with an exponential time course.

Although studies on the isotonic velocity transients provided considerable insights into the mechanism producing force and motion in muscle, it has not been possible to decide which or which part of these models are correct. Next, we will consider other kinds of approaches to the understanding of muscle contraction mechanism.

3. ISOMETRIC FORCE TRANSIENTS

Another effective means to obtain information about the mechanism of muscle contraction is to apply step changes in length to an isometrically contracting muscle fiber.

In frog muscle fibers, however, the sarcomere spacings tend to be shorter and less extensible near the fiber ends than at the middle region where the sarcomere spacings are reasonably uniform. Consequently, the applied length changes does not take place uniformly along the fiber length. To overcome this difficulty, Gordon et al. (1966 *a*, *b*) developed ingenious spot-follower method, with which fiber length was controlled at the middle part between the two position markers on the fiber. With this method, they could establish a well-known sarcomere length-force relation, which has been generally taken as evidence that the cross-bridges are independent force generators.

Huxley and Simmons (1971) also used the spot-follower method to study the isometric force transients in response to step decreases in fiber length with results and interpretations illustrated in Fig. 3. They interpreted the initial force drop coincident with the length step (from T_0 to T_1, Fig. 3A) as being due to the cross-bridge elasticity, while they interpreted the initial quick force recovery (from T_1 to T_2) as being due to rotation of the cross-bridges that remain attached to the thin filament either before and after the length step. Huxley and Simmons proposed a model diagrammatically shown in Fig. 3B, C. Here, the cross-bridge (M) and the spring connecting it to the myosin filament (S-2) are drawn more realistically than Huxley's 1957 model. Both M and the thin filament are assumed to have more than one combining site (for example M_1 to M_4 and A_1 to A_4, Fig. 3B). M first attaches to A by forming links M_1A_1 and M_2A_2, while the spring is at its slack length (Fig. $3C_1$). Then, according to the assumption of the potential energy diagram along they attachment sites A_1 to A_4, M changes its attachment sites from M_1A_1-M_2A_2 to M_2A_2-M_3A_3 and then to M_3A_3-M_4A_4. As a result, M rotates along the thin filament to pull the spring S-2, and the resulting force in S-2 shows up as isometric force if the thick and thin filaments are not allowed to slide past each other, or it causes the relative filament sliding. The ATP splitting is assumed to be coupled with the detachment of M from A. A quick release applied to an isometrically tetanized fiber causes a decrease in force in the spring, resulting in a quick force drop from T_0 to T_1 (Fig. 3A). This is followed by the increased probability that the attached M rotates on the thin filament to pull back the spring, resulting in the quick force recovery (phase 2). The subsequent slow force recovery is due to turnover of A-M links.

The main difference between the Huxley-Simmons model and the Huxley 1957 model is that, in the former, there are more than one A-M links, including the initial nonforce-generating state, while in the latter the A-M link has only one state and generates force as soon as A-M link is formed. The presence of the nonforce-generating A-M link is in accord with the phenomenon that both the stiffness increase and the transfer of mass from the thick to the thin filament as evidenced by the change in equatorial X-ray diffraction pattern (for a review. see Huxley and Faruqi 1983) occur ahead of the force development (see, Fig. 4A). The Huxley-Simmons model has been useful in relating the hypothetical A-M links to the enzyme kinetics of actomyosin ATPase in solution. The amount of extension of the spring S-2, when the force in a myosin head increases to the maximum value T_0, has been carefully estimated by A. F. Huxley and his coworkers (Ford et al. 1977) to be less than 4 nm.

The only experimental evidence for the cross-bridge origin of the T_1 and T_2 curves was that these curves were scaled according to the number of attached cross-bridges. If the steady isometric force P_0 was altered by changing the amount of filament overlap, the curves relating the level of T_1 and T_2 to the shortening step (T_1 and T_2 curves) were scaled according to the amount of P_0. Both T_1 and T_2 curves were not, however, obtained up to the level of zero force, since the feedback loop in the spot-follower did not

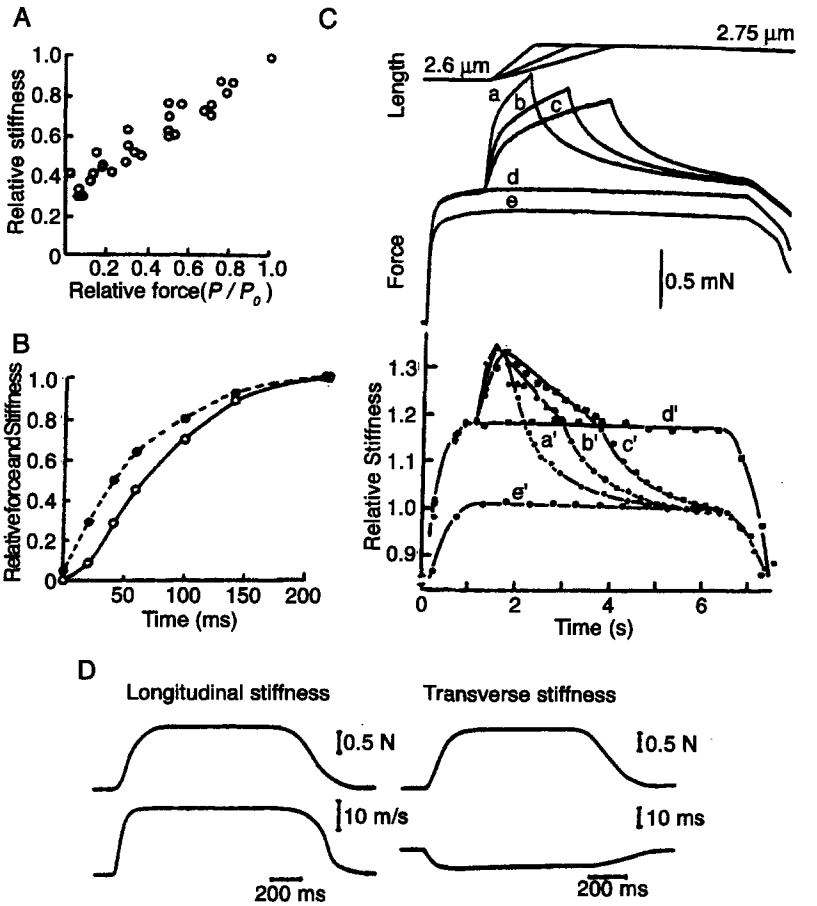

Figure 4. Muscle and muscle fibers stiffness changes in various experimental conditions. (A) Dependence of muscle fiber stiffness on the amount of load (= force) during steady isotonic shortening (Julian and Sollins 1975). (B) Increase in muscle fiber stiffness (*filled circles*) and force (*open circles*) during rise of isometric teatnic force (Cecchi et al. 1984). (C) Force and stiffness changes of a tetanized fiber in response to slow stretches. The fiber was first tetanized isometrically at 2.6 μm sarcomere length, and then slowly stretched to 2.75 μm sarcomere length with three different velocities. *Upper, middle* and *lower traces* show time course of changes in the fiber length, the force and the stiffness, respectively. Traces a, b and c in the force records correspond to traces a', b' and c' in the stiffness trace, respectively. Traces d and e are records of ordinary isometric forces at sarcomere length of 2.6 μm to 2.75 μm, respectively. Note that the stiffness increases abruptly on application of stretch, then decrease linearly during stretch, and eventually decreases to a steady level equal to that of ordinary isometric tetanus at a sarcomere length of 2.75 μm after completion of stretch, whereas the isometric force attained after completion of stretch is appreciably higher than the ordinary isometric force at a sarcomere length of 2.6 μm despite the decrease in the amount of myofilament overlap (Sugi and Tsuchiya 1988). (D) Changes in the force (*upper traces*) and the stiffness (*lower traces*) of a muscle during isometric tetanus . The stiffness was measured as propagation velocity of ultrasonic waves (3-7 MHz) between the two piezoelectric transducers. Upward and downward deflections of the stiffness records represent increase and decrease of stiffness, respectively. Note that the transverse stiffness decrease during contraction (Hatta et al. 1988).

work well at low force range, where the slope of length-step versus force relation was not steep. Therefore, the evidence for the validity of the model was incomplete. Meanwhile, Huxley and Simmons (1971) used very rapid length steps and examined the resulting force transients on a very fast time base, so that the force in the fiber was not uniform in every fiber cross-section; in other words, it was impossible in principle to actually record and control "segmental force" in the length-controlled segment. In fact, Sugi and his colleagues have demonstrated with various methods, including ultrahigh-speed cinematography and ultrahigh sensitivity sarcomere length measurement, that there are considerable nonuniformities in both length and force during the period of quick force recovery (Sugi and Kobayashi, 1983), indicating that the quick force recovery does not reflect fundamental molecular events responsible for force and motion in muscle. Now, it is generally agreed that the series elastic component originates not only from the cross-bridges but also from the thin filament (for a review, see Sugi and Tsuchiya 1998), while no concrete evidence has been obtained for the cross-bridge rotation during muscle contraction (for reviews, see Sugi, 1992, 1993; Sugi and Tsuchiya, 1998).

4.MUSCLE FIBER STIFFNESS

In the Huxley contraction model, in which myofilaments are assumed to be rigid, small length perturbations (~ 0.1 % of L_0, kHz range) applied to a tetanized muscle fiber would be taken up by the elastic link connecting each cross-bridge to the thick filament in both positive and negative x regions (Fig. 1). Thus the resulting force changes serve as a measure of total number of attached cross-bridges, if the compliance of recording system are reasonably reduced. Julian and Sollins (1975) measured muscle fiber stiffness by applying such small length perturbations during steady isotonic shortening, and showed that the stiffness decreased with decreasing isotonic load (= force), approaching a minimum value (about 20 % of the maximum value at the plateau of isometric tetanus) as the load tended to zero (Fig. 4A). This result is in fairly good agreement with the Huxley contraction model with respect to the total number of attached cross-bridges at various steady shortening velocities (Fig. 1B, C). On the other hand, the fiber stiffness rises from zero to the maximum value during isometric force development, reflecting an increase in the number of attached cross-bridges. Cecchi *et al.* (1982, 1986) have shown that muscle fiber stiffness rises ahead of force by more than 15 ms (Fig. 4B) during isometric force development (Fig. 4B). This can be taken to indicate that, as predicted by the Huxley-Simmons model, cross-bridge first attaches to the thin filament, and then undergoes structural changes to produce force after a definite delay.

Sugi and Tsuchiya (1988) have demonstrated a marked dissociation between the stiffness and the isometric force when an isometrically tetanized fiber is subjected to a slow stretch. As illustrated in Fig. 4C, the stiffness first rises to a steady level as the fiber develops a steady isometric force on tetanic stimulation. On application of a slow stretch at the plateau of the preceding isometric tetanus, the stiffness shows an abrupt rise (probably due to synchronized extension of attached cross-bridges) which is then followed by a gradual decrease to a new steady level appreciably below the level of the preceding tetanus, reflecting a decrease in the amount of myofilament overlap caused by the stretch. The marked decrease of the stiffness starts when the stretch is still going on and the force is rising, and the final stiffness level attained after the end of stretch is

definitely below the initial stiffness level, although the isometric force attained after the end of stretch is appreciably higher than the normal isometric force at the stretched fiber length. These results indicate that cross-bridge elasticity may differ depending on their physiological states.

The stiffness measurements described so far are concerned with relative values expressed as the magnitude of force changes caused by small constant length changes in kHz range. Sugi and his coworkers (Tamura *et al.*, 1982; Hatta *et al.*, 1988) developed a method, with which muscle stiffness was measured with ultrasonic waves in MHz region, with negligibly small perturbations to the muscle. The stiffness measurement is based on the equation,

$$V_f = (C_f/\rho)^{1/2},$$

where V_f is the phase velocity of ultrasonic waves, C_f is the muscle stiffness, and ρ is the density of muscle; this is essentially the measurement of propagation velocity of ultrasonic waves in muscle. The advantage of this method is that the stiffness can be measured not only along the muscle length as with other stiffness measurements (longitudinal stiffness), but also across the muscle transverse section (transverse stiffness). On tetanic stimulation, the longitudinal stiffness rises ahead of force by more than 20 ms during isometric force development, while it lags behind the force during relaxation of isometric force (Fig. 4D, left). On the other hand, the transverse stiffness decreases during isometric tetanus (Fig. 4D, right). This interesting phenomenon, possibly reflecting change in the state of water between the myofibrils as ATP hydrolysis goes on, should further be studied using single fibers. On the other hand, both the longitudinal and transverse stiffness remained unchanged if a muscle in rigor state was stretched by 1 - 2 %, so that the rigor force increased twofold. If the ultrasonic waves are assumed to shake water molecules, the state of water may change dynamically during active force generation but not in rigor state.

Evidence has been accumulating that, muscle fiber stiffness measured with step length changes over a wide range of velocities extending above the kHz range, exhibit feature completely different from those obtained with kHz range perturbations (Bagni et al, 1992, 1995; Stehle and Brenner, 2000). This is not surprising, since force generation in muscle is a dynamic phenomenon involving changes in small molecules, macromolecules, myofilaments and higher structures; the above hierarchy in muscle structure responsible for contraction would exhibit different features according to frequency of externally applied perturbations.

5. THE DISTANCE OF CROSS-BRIDGE POWERSTROKE

The distance cross-bridge powerstroke d is defined as the distance, over which a cross-bridge attached to the thin filament exerts positive force to produce force and motion utilizing energy derived from ATP hydrolysis. On the basis of Huxley-Simmons model, d corresponds with the distance of myofilament sliding achieved by the cross-bridge rotation (Fig. 3C). Since the myosin head is pear-shaped with the larger diameter of ~ 20 nm, it gives a structural constraint that d is ~ 10 nm and never more than 20 nm. The development of *in vitro* motility assay system, in which fluorescently labeled actin filaments are made to slide on myosin fixed to a glass surface (Kron and Spudich, 1986), has made it possible to roughly estimate the value of d by measuring the

amount of ATP utilized and the distance of actin filament sliding on myosin, assuming *d* in each cross-bridge is coupled with hydrolysis of one ATP molecule. Due to lack of accuracy in measuring the amount of ATP utilized, however, the estimated values of cross-bridge powerstroke ranged from ~ 8 nm to > 200 nm (Toyoshima et al., 1990; Harada et al., 1990), and no definite conclusion has been reached with this kind of approach.

On the other hand, a number of motor proteins other than muscle myosin, which is now called myosin II, have been found, and their motor functions have been compared using the in vitro motility assay systems. Based on both density of motor proteins on a glass surface and the minimum length of actin filaments (or microtubules) to move on myosin without being detached, the concept of "duty ratio" , i.e. the fraction of time in which a motor protein is attached to its filament, has proved to be useful in understanding functional diversity of motor proteins (for a review, see Howard, 1997).

The duty ratio of skeletal muscle myosin is regarded to be small, being 0.01 - 0.1 (Uyeda et al., 1990; Leibler and Huse, 1993). In terms of the duty ratio *P*, the value of *d*, coupled with hydrolysis of one ATP molecule, can be expressed as:

$$P = (d \bullet A) / V_{max} \quad (1),$$

where A is ATPase rate per cross-bridge and V_{max} is the maximum velocity of actin-myosin sliding under zero external load. If we put 20 s^{-1} cross-bridge^{-1} (Toyoshima et al., 1987) for A and ~ 3 mms^{-1} (Oiwa et al., 1990) for V_{max} in equation 1, a value of *d* of 4 - 16 nm, consistent with the Huxley-Simmons model (1971), can be obtained. This of course does not necessarily prove the validity of equation 1 in a muscle fiber shortening with V_{max}; in the Huxley contraction model, in which a huge number of cross-bridges are interacting with the thin filaments, the cross-bridges, once attached to the thin filament in the positive *x* region (Fig. 1A), is brought into the negative *x* region before being detached from the thin filament. Based on the Huxley model, Higuchi and Goldman (1991) attempted to determine the interaction distance of cross-bridges *Di*, extending over both positive *x* and negative *x* regions. The equation they used is:

$$Di = Ds\ Sa\ [M_0] / [ATP_u] \quad (2),$$

where Ds is the total distance of myofilament sliding, Sa is the average muscle fiber stiffness during shortening (expressed relative to rigor stiffness), $[M_0]$ is the cross-bridge concentration in the fiber (154 μM), and $[ATP_u]$ is the ATP concentration utilized for fiber shortening. They used the technique of laser-flash photolysis of caged ATP to induce shortening of a demembranated muscle fiber. The value of Di obtained was ~ 28 nm. This experiment includes many uncertainties in the values to be put in equation 2, especially S_a, and gives no direct information about the value of *d*.

To determine the distance of cross-bridge powerstroke without measuring muscle fiber stiffness, which appears to be an incomplete measure of the number of working cross-bridges, Yamada et al. (1993) used the techniques of laser-flash photolysis of caged ATP and high-speed video recording of the whole demembranated muscle fiber. Under nearly isometric conditions in which the cross-bridges might only exist in the positive x region according to the Huxley contraction model (Fig. 1), they found that the minimum uniform ATP-induced fiber shortening was ~ 10nm per half sarcomere, a value which can be regarded as *d* within the framework of Huxley contraction model (Fig. 5). Using an in vitro motility assay system, in which a myosin-coated microneedle was made to slide along actin filaments by iontophoretically applied ATP (Oiwa et al., 1993), the unitary actin-myosin sliding (analogous to *d*) was also found to be ~ 10 nm.

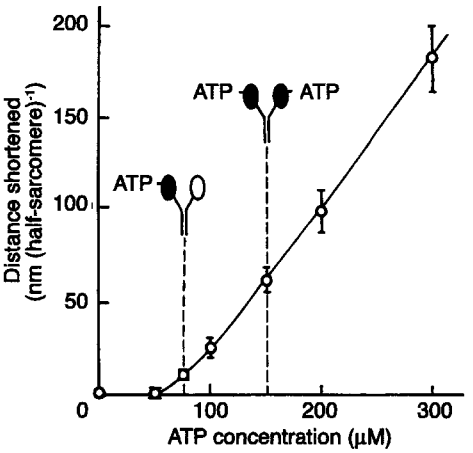

Figure 5. Relation between the concentration of released ATP and the velocity and distance of muscle fiber shortening. The initial shortening velocity and the distance shortening are plotted against the ATP concentration in A and B, respectively. Vertical dashed lines and diagrams of myosin molecules show the ATP concentrations equal to, and a half of, the total myosin head concentration within the fiber (150 μM) (Yamada et al., 1993).

On the other hand, the most straightforward way to measure the distance of cross-bridge powerstroke is to observe living muscle thick filaments under an electron microscopy (magnification, x 10,000) using a gas environmental chamber, with the filaments can be kept alive by circulating water vapor (Fubani et al.), and record ATP-induced movement of cross-bridges on the thick filament. By putting position markers on the cross-bridges on the cross-bridges extending from the synthetic thick filaments (myosin-paramyosin core complex), Sugi et al. (1997) have succeeded in recording the cross-bridge movement induced by iontophoretically applied ATP. Their results are summarized as follows (Fig. 6):

1. The mean position of each cross-bridge does not change with time despite its thermal motion.

2. In response to ATP, the cross-bridges move along the fiber length. The mean distance of the ATP-induced cross-bridge movement is ~ 30 nm, a value larger than the structural constraint of cross-bridge powerstroke (Fig. 6).

3. The cross -bridges does not move in response to ADP.

4. As the experimental system does not contain actin filaments, the above results indicate most of the cross-bridge motion can take place with our their attachment to actin.

At present, the observed cross-bridge movement is likely to be a recovery stroke preceding the powerstroke. The distance of cross-bridge motion of ~ 30 nm can only be accounted for by assuming shortening of the subfragment-2 region (length, ~ 43 nm), connecting the myosin head to the thick filament backbone (Harrington et al., 1988; Sugi et al., 1992). We are now preparing the experiments, in which synthetic bipolar myosin filaments are made to slide past actin filaments, in the hope to clarify mysteries about the cross-bridge powerstroke.

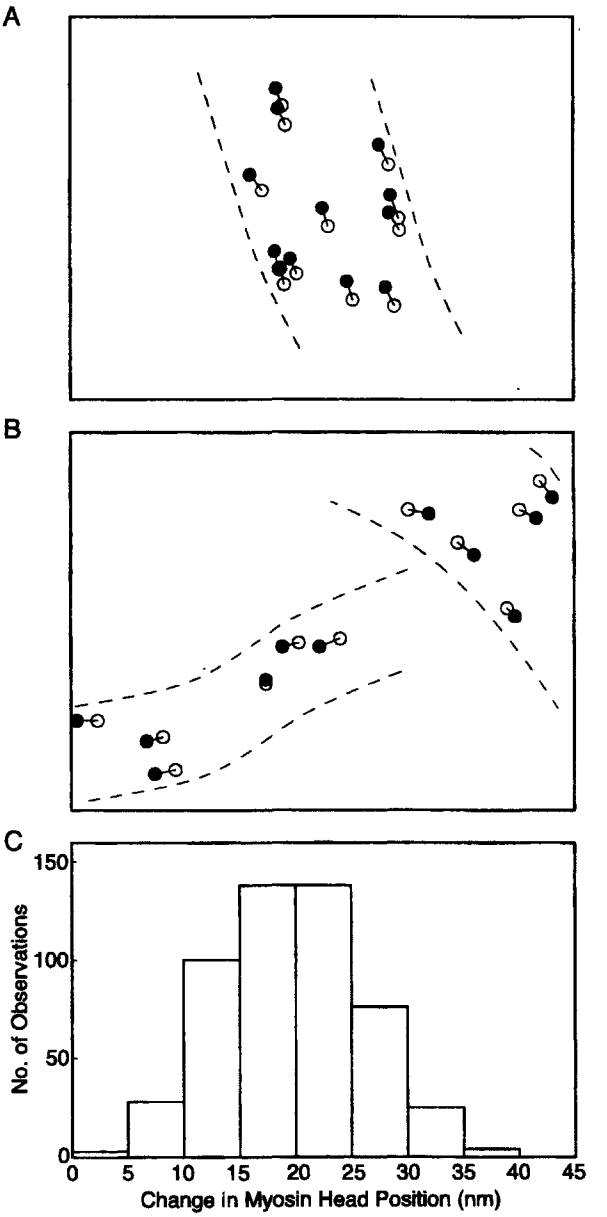

Figure 6. Movement of the individual myosin heads on the filaments in response to ATP application. (A and B) Examples showing changes in the center of mass position of the same particles after ATP application. Filled and open circles (diameter, 15 nm) are also drawn around the center of mass positions of the same particles in the records before and those after ATP application, respectively (Sugi et al., 1997).

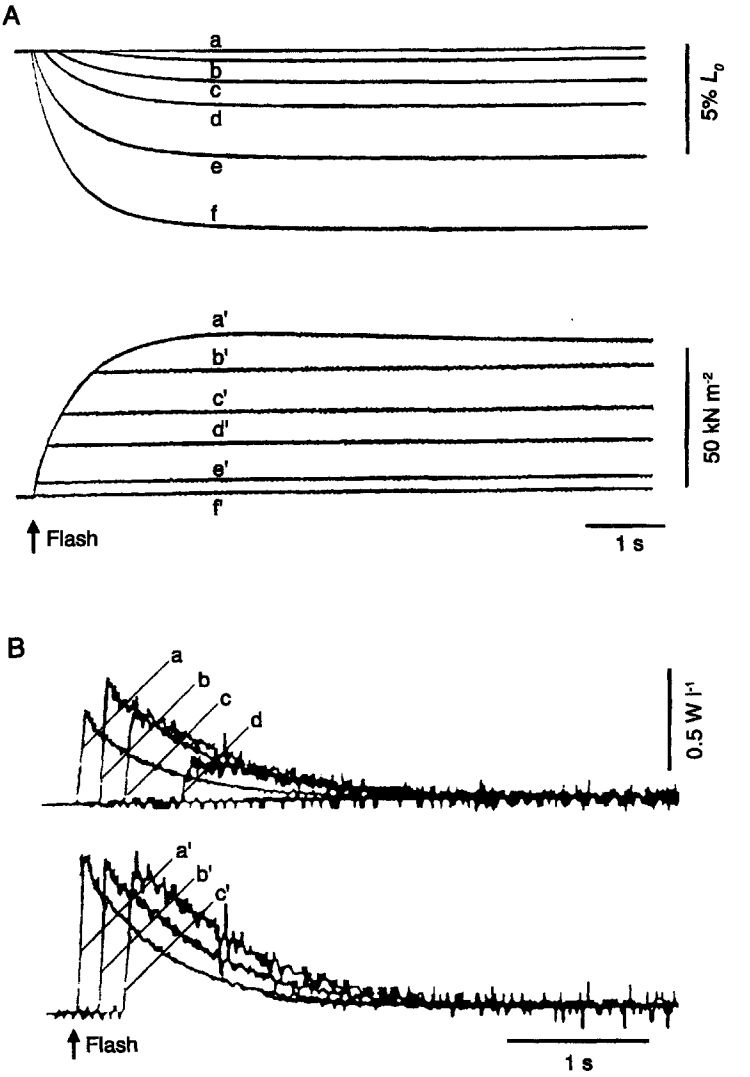

Figure 7. (A) Laser flash-induced mechanical response. Upper and lower traces show fiber length and force changes, respectively. Length recordings a, b, c, d, e and f correspond to force recordings a', b', c', d', e' and f', respectively. The loads were P_0 (isometric contraction *a, a'*), 0.78 P_0 (*b, b'*), 0.53 P_0 (*c, c'*), 0.35 P_0 (*d, d'*), 0.09 P_0 (*e, e'*), and 0 (unloaded shortening, *f, f'*), respectively. (B) Power output during flash-induced fiber shortening. Upper traces: Power output recordings under four different afterloads. Lower traces: Power output records normalized relative to the peak values attained. The load was 0.09 P_0 (*a, a'*), 0.35 P_0 (*b, b'*), 0.53 P_0 (*c, c'*) and 0.78 P_0 (*d*). The recordings were obtained from the experiment shown in Fig. 1 (Sugi et al., 2003).

Apart from the experiments described above, an idea of "biased Brownian ratchet mechanism" has been proposed to account for some in vitro motility experiments,

suggesting *d* values well beyond the structural constraints of the cross-bridge (Vale and Oosawa, 1990). I wonder how this idea harmonizes with the vast physiological results accumulated in the past. In my feeling, the criteria for "single molecule events", obtained from experiments, in which a single myosin molecule on a latex bead is made to interact with a single actin filament, are not strict among the motility assay people, and may be associated with a number of pitfalls, considering the bead diameter ~ 10^6 times larger than the size of a myosin head.

6. THE MECHANICAL EFFICIENCY OF CROSS-BRIDGE POWERSTROKE

The mechanical efficiency, with which chemical energy derived from ATP hydrolysis is converted into mechanical work, in demembranated muscle fibers has now been estimated by measuring the amount of ATP utilized for work production, using fluorescence of a phosphate-binding protein (He et al., 1999) or NADH (Reggiani et. al., 1997; Sun et al., 2001). During myofilament sliding, however, the cross-bridges not only attach to the thin filament in the positive force region to perform their powerstroke-producing positive forces, but also produce negative forces in the negative force region before being detached from the thin filament (Fig. 1). On this basis, the overall mechanical efficiency of muscle fibers may be much smaller than that of individual cross-bridges during their powerstroke, since positive forces are always opposed by negative forces, due to asynchronous cross-bridge activity. To accurately estimate the mechanical efficiency of individual cross-bridges when they perform their powerstroke, it is necessary to perform experiments under condition in which the cross-bridges start their powerstroke synchronously.

For this purpose, we used an experimental condition, in which each cross-bridge (= myosin head) in the demembranated muscle fiber could hydrolyze ATP only once during a single mechanical response (Sugi et al., 1998, 2003). This condition is achieved by soaking the fibers in a solution containing ATP at a concentration almost equal to the total myosin head concentration in the fiber, exposing them in air to blot solution around the fibers, and then rapidly activating them to contract by using laser flash photolysis of caged calcium. Immediately before activation, the number of ATP molecules in the fiber is made almost equal to the number of myosin heads, so that all the myosin heads are in the state M•ADP•Pi, in which ATP is already hydrolyzed by the myosin head (M) but its products are still bound to M (Bagshaw, 1984).

When the fibers are maximally activated by photo-released Ca^{2+}, they first develop an isometric force equal to the afterload P, start shortening isotonically, and then eventually stop shortening as the fibers enter into rigor state after complete exhaustion of ATP (Fig. 7A). As shown in Fig. 7B, the power output in the flash-activated fibers reached a peak at the early phase of fiber shortening, and then decreased with time. The higher the initial peak, the larger the area under the power output trace, i.e. the amount of work done by fiber shortening. The power output records were almost identical when normalized with respect to their peak values, except for the load close to P_0. The distance of fiber shortening, when the power output reached a maximum did not exceed 10 nm per half sarcomere. This suggests that, at the beginning of fiber shortening, the cross-bridges, in the form of M•ADP•Pi, start their powerstroke almost synchronously, while sensing the amount of load to determine their energy output.

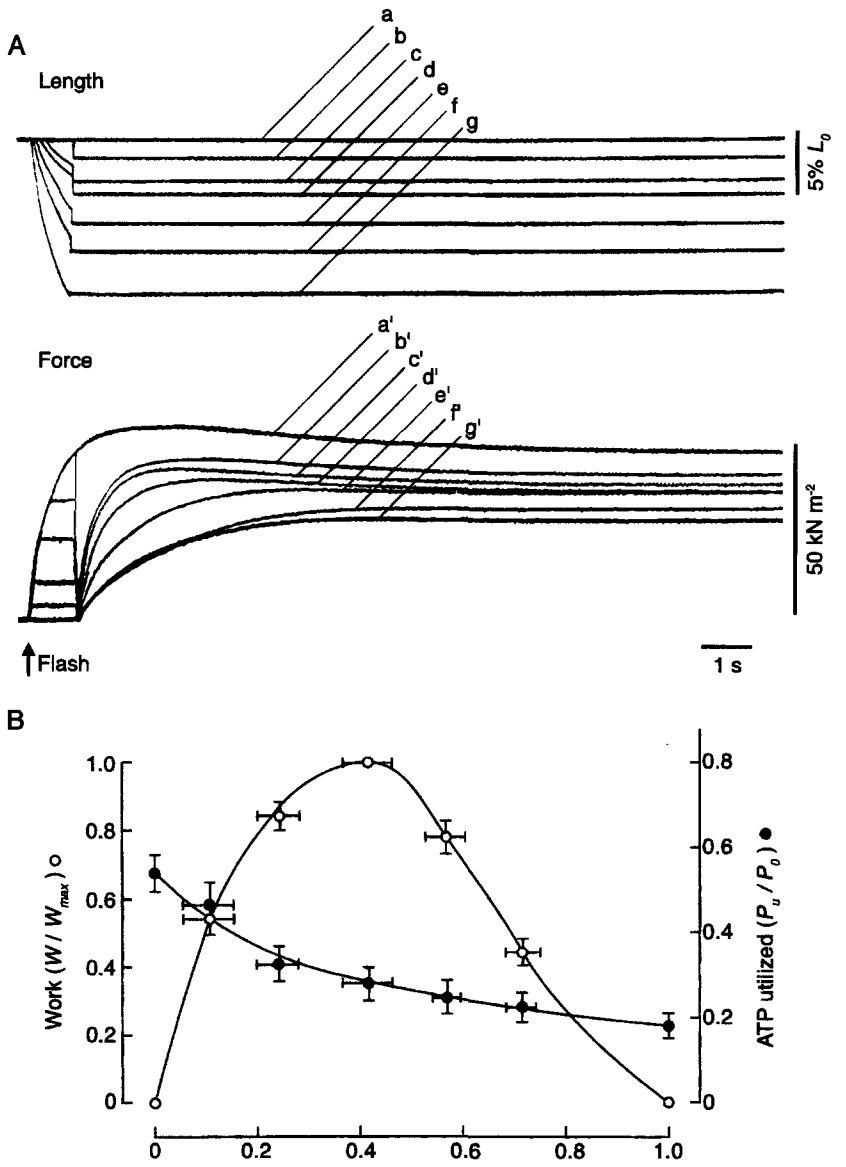

Figure 8. (A) Fiber length and force changes of a preparation that was first made to shorten isotonically under five different afterloads, and then subjected to quick releases at 1s after activation to drop the force to zero. After each release, the preparation redeveloped isometric force at the decrease fiber length. Length recordings *a* – *g*, correspond to force recordings *a'* – *g'*, respectively. The load was P_0 (isometric contraction *b*, *b'*), 0.63 P_0 (*c*, *c'*), 0.41 P_0 (*d*, *d'*), 0.20 P_0 (*e*, *e'*), 0.09 P_0 (*f*, *f'*), and 0 (unloaded shortening, *g*, *g'*). Recordings *a*, *a'* were obtained during isometric contraction without quick release. (B) Dependence of the amount of ATP utilized for mechanical response (*P*u, filled circles) and the amount of work done (*W*, open circles) on the isotonic load (*P*) at 1 s after flash activation. Values were means ± S.E.M. obtained from eight different data sets (Sugi et al., 2003).

During isometric contraction, in-phase stiffness, i.e. the magnitude of force changes in response to sinusoidal length changes, increased approximately in parallel with isometric force, while quadrature stiffness, i.e. the 90° out-of phase stiffness component, reached a maximum at approximately 0.3s after activation, and stayed almost unchanged for the following 3-4 s. This indicates that there were no appreciable changes in the number of force-generating cross-bridges during isometric force development preceding fiber shortening, since quadrature stiffness is taken as a measure of the fraction of active cross-bridges (Goldman et al., 1984). Furthermore, under conditions identical to the present experiments, no appreciable increase of internal resistance against fiber shortening takes place at least for the first 1-2 s after activation (Sugi et al., 1998). It may therefore be safe to conclude that, at least for 1-2 s after activation, the cross-bridges may not readily from rigor links after releasing Pi and ADP, irrespective of whether the fiber is shortening or kept isometric.

Fig. 8A shows a typical experiment in which the fibers were activated to contract isometrically or isotonically under five different afterloads for 1s, and then subjected to a quick release to drop the force to zero, whereon the fiber length was clamped and the fibers developed after a quick release (P_r), i.e. a measure of the amount of ATP remaining in the fiber at 1 s after activation, was maximum when $P = P_0$ (isometric contraction and minimum when $P = 0$ (unloaded shortening). Similar results were obtained from 7 different preparations examined. The amount of ATP utilized at 1 s after flash activation ($P_u = P_0 - P_r$) was therefore maximum during unloaded shortening (P = 0), and minimum during isometric contraction ($P = P_0$).

On the other hand, the possibility that cross-bridges forming rigor links with the thin filaments may produce rigor force to contribute to the isometric force development after a quick release can be precluded by the extremely slow development of rigor force in demembranated muscle fibers (Kobayashi et al., 1998).

Fig. 8B shows the dependence of the amount of work done (W, expressed relative to the maximum value, W_{max}) and the amount of ATP utilized for the whole mechanical response (P_u, expressed relative to P_0) on the isotonic load (P). The data points were obtained from 8 different datasets. The value of P_u at P = 0 was approximately 3 times larger than that at $P = P_0$.

The amount of ATP utilized for the whole mechanical response (P_u) is the sum of the amount of ATP utilized for the preceding isometric force development (P_i) and that utilized for subsequent isotonic shortening (P_s). The value of P_i as a function of isotonic load were obtained by applying a quick release to isometrically contracting fibers at various times after activation and measuring the amount of force development after each quick release. Thus, the value of P_s could be obtained by subtracting the value of P_i for a given isometric force equal to the isotonic load from P_u for the whole mechanical response. The mechanical efficiency of individual cross-bridges (E), averaged over the period of work production, can be estimated as $E = W / (P_u - P_i) = W / P_s$. The dependence of E (expressed relative to the maximum value, E_{max} on the isotonic load is shown schematically in Fig. 9 together with W, P_u, P_i and P_s. The value E versus P relationship was bell-shaped, with a broad peak at $0.5 - 0.6\ P_0$.

Although the mechanical efficiency of individual cross-bridges is obtained as relative values in the present study, we made a conservative estimation of its absolute value as follows. The average fiber cross-sectional area of 8 preparations, from which the data shown in Fig. 4 were obtained, was $6.1 \pm 0.1 \times 10^{-5}$ cm^2, while the fiber length was $\leq 2.5 - 3$ mm. To avoid underestimation of fiber volume leading to overestimation

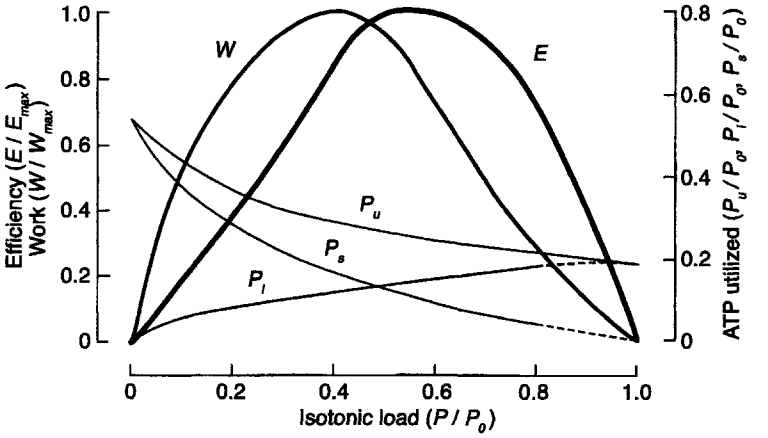

Figure 9. Dependence of the mechanical efficiency of individual cross-bridges (*E*) on the amount of isotonic load (*P*) obtained from the results shown in Figs 4 and 5. Values are scaled to adjust the value at 1.0 P / P_0 to that in Fig. 4. The amount of work done (*W*), ATP utilized for preceding isometric force development (*Pi*), and ATP utilized for isotonic shortening (*Ps*) are also shown as function of load (Sugi et al., 2003).

of the efficiency, we use the maximum fiber length 3 mm to obtain mean fiber volume of 1.8×10^{-5} cm^3. Assuming a cross-bridge concentration of 200 _ $moll^{-1}$ (higher than the widely used values of 145 or 150 μmol^{-1}), the amount of M•ADP•Pi immediately before flash activation is estimated to be 3.6×10^{-6} μmol ($200 \times 1.8 \times 10^{-5} \times 10^{-3}$) = 3.6×10^{-12} mol. In Fig. 7, the value of E is maximum at $P = 0.53\ P_0$, and the corresponding value of P_s is $0.13\ P_0$, where corresponds to the initial amount of M•ADP•Pi of 3.6×10^{-12} mol. The number of ATP molecule utilized for work production is calculated to be. 2.8 $\times 10^{11}$ ($3.6 \times 10^{-12} \times 0.13 \times 6 \times 10^{23}$). Assuming the energy released by ATP hydrolysis of 50 $kJmol^{-1}$ (Bagshaw, 1984; Oiwa et al., 1991), the energy available from one ATP molecule is 8.3 x_10^{-20} J (50 x_10^3 / 6 x_10^{23}). The energy released from ATP molecules during work production is 2.3 x_10^{-8} J (2.8 x_10^{11} x 8.3 x_10^{-20}). In Fig. 6, the amount of work done at 0.53 *Po* is 1.6 x_10^{-8} J. The maximum mechanical efficiency of individual cross-bridges is therefore estimated to be (1.6 x_10^{-8})/ (2.3 x_10^{-8}) = 0.7. Since the above estimation is conservative, the actual maximum mechanical efficiency of individual cross-bridges is suggested to be 0.8 - 0.9, being close to unity.

These results may constitute evidence that the maximum mechanical efficiency of individual cross-bridges may be very high, probably close to unity (0.8 – 0.9). In this connection, it is of interest to note that it has also been suggested that the mechanical efficiency of the ATP-dependent rotary motion of F_0-F_1 ATPase at the mitochondrial membrane is close to unity (kinoshita et al., 2000).

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DISCUSSION

Maughan: Prof. Sugi, your elegant ultrasonic wave (MHz) experiments are very impressive. Does this technique prove movement of small molecules, including water? Do you in fact believe water is the major molecule probe?

Sugi: Yes, I've discussed the results with Andrew Huxley and he agrees with me.

Maughan: Thus, could the MHz signal measure crossbridge movement indirectly by its action of pushing water during its movement?

Sugi: I believe that, with ultrasonic waves, we are shaking water molecules in between the myofilaments. Water molecules are known to undergo dynamic changes during

contraction. It should be noted that the MHz wave stiffness is insensitive to resting tension, indicating that the MHz waves do not detect changes in strain of myofilaments. In addition, the MHz stiffness in rigor fibers do not change by stretch despite a great increase in rigor force, again indicating that the MHz stiffness does not detect strain of cross-bridge.

Pollack: Your idea that the MHz stiffness changes reflect the state of water is consistent with the ^{1}H NMR studies of Yamada et al. (Yamada, Biochim, Biophys. Acta 1379: 224-232, 1998). Several people in the audience (Drs. Yamada and Ogata) have reported changes in water structure during the transition from rest to activity. Therefore it seems that your hypothesis may be correct: the difference of ultrasonic velocity may well be related to the state of water.