brief communication

Covalent cross-linking of single fibers from rabbit psoas increases oscillatory power

Katsuhisa Tawada* and Masataka Kawai‡
*Department of Biology, Faculty of Science, Kyushu University, Fukuoka 812, Japan; and ‡Department of Anatomy, College of Medicine, University of Iowa, Iowa City, Iowa 52242

ABSTRACT Single fibers from chemically skinned rabbit psoas muscle were treated with 1-ethyl-3-[3-(dimethyl-amino)proyl]-carbodiimide (EDC) at 20°C after rigor was induced. A 22-min treatment resulted in 18% covalent cross-linking between myosin heads and the thin filament as determined by stiffness measurements. This treatment also results in covalent cross-linking among rod portions of myosin molecules in the backbone of the thick filament. The fibers thus prepared are stable and do not dissolve in solutions at ionic strengths as high as 1,000 mM. The preparation was subjected to sinusoidal analysis, and the resulting complex modulus data were analyzed in terms of three exponential processes, (A), (B), and (C). Oscillatory work (process B) was much greater in the cross-linked fibers than in untreated ones in activating solutions of physiological ionic strength (200 mM); this difference was attributed to the decline of process (A) with EDC treatment. Consequently, the Nyquist plot of the EDC-treated preparation exhibited an insect-type response. We conclude that, under these conditions, both cross-linked and non-cross-linked myosin heads contribute to the production of oscillatory power. The cross-linked preparations also exhibited oscillatory work in high ionic strength (500–1,000 mM) solutions, indicating that cross-linked myosin heads are capable of utilizing ATP to produce work. We conclude that process (A) does not relate to an elementary step in a cross-bridge cycle, but it may relate to dynamics outside the cross-bridge such as filament sliding or sarcomere rearrangement.

INTRODUCTION

It has been known for some time that, in active muscle, myosin cross-bridges cyclically interact with actin to transduce chemical energy stored in ATP into useful work (Huxley, 1974; Eisenberg and Hill, 1985). When the muscle is allowed to shorten, it performs work on the external environment. Whereas it is generally believed that one cross-bridge cycle is accompanied by the hydrolysis of one ATP molecule, recent evidence suggests that several cross-bridge cycles may be involved in the hydrolysis of one ATP molecule (Yanagida et al., 1985). Thus, it is useful to study cross-bridges which are made unable to detach from thin filaments, and to ask whether these cross-bridges are capable of hydrolyzing ATP and producing work. To answer these questions, we used single fibers from chemically skinned rabbit psoas muscles, and performed covalent cross-linking between the myosin head and actin, as initially tried in an isolated protein system by Mornet et al. (1981). The current results were presented at a recent Biophysical Society Meeting (Kawai and Tawada, 1990).

MATERIALS AND METHODS

Chemically skinned rabbit psoas fibers were prepared as reported (Kawai and Halvorson, 1989). Single fibers were dissected and mounted in a device that changes the length and detects tension of the fibers. The ends of the fibers were wrapped into specially designed clamps, and fibers were secured by No. 6 silk thread which was untwisted and teased. The sarcomere length was adjusted to 2.4 μm. The relaxing solution for mounting a segment of muscle fibers contained (in millimolars) 2 MgATP, 5 EGTA, 10 MOPS (pH 7.0), and ionic strength was adjusted to 200 mM with K propionate. Rigor was induced by replacing saline with a solution that contained (in millimolars:) 80 KCl, 40 imidazole, 7.4 EDTA (pH 7.00). This produces the "low rigor" state described earlier (Kawai and Brandt, 1976). After the induction of rigor, the length (L_o) and the diameter of each fiber were measured, and the preparation was treated for 22 min at 20°C with a cross-linking solution which contained 10 mM 1-ethyl-3-[3-(dimethyl-amino)proyl]-carbodiimide (EDC), 80 KCl, 40 imidazole (pH 7.00). The cross-linking solution was continuously circulated within the muscle chamber to avoid local heterogeneities in EDC concentration and temperature. The EDC reaction was quenched by several washes with 0.2% (vol/vol) beta mercapto-ethanol added to the rigor solution, followed by immersion in this solution for 10 min. The solution was then replaced with rigor solution, and the preparation was repetitively stretched/released by 0.7% L_o for 60 min at a frequency of 1–2 stretches per 2 s, so that any weak cross-links were removed. This procedure typically cross-links rods of myosin molecules covalently in the thick filament backbone so that sarcomeres are no longer dissolved in high ionic strength solutions (500–1000 mM; Tawada and Kimura, 1986). This procedure also cross-links ~20% of myosin heads covalently to actin (Tawada and Kimura, 1986).

The degree of cross-links was assessed by stretching the fibers by 0.20, 0.35, 0.50, and 0.65% L_o, and by detecting fiber stiffness (Tawada and Kimura, 1986). As reported previously, stiffness was the same for any stretch up to 0.65% L_o, a result of the high degree of linearity in the tension responses to length steps. The stiffness in high ionic strength

Address correspondence to Dr. Kawai.

(500 mM) relaxing solution was compared with that in rigor solution at pH 7.00 (the relaxing solution contained in millimolars, 6 EGTA, 5.3 MgATP, 4.7 ATP, 8 Pi, 15 CP, 32 KProp, 26 NaProp, 300 KCl, 10 MOPS, 160 U/ml CK; the rigor solution contained 8 Pi, 76 NaProp, 103 KProp, 300 KCl, 10 MOPS).

The preparations were subjected to small amplitude (0.25% peak-to-peak) oscillations at 18 discrete frequencies between 0.25 and 350 Hz. The tension and length signals were digitized, signal averaged, and the complex modulus data (defined as the ratio of stress to strain in the frequency domain) were calculated. The data were corrected against the system response by using the response of the rigor preparation as an ideal elastic material. The frequency profile of the corrected complex modulus data $Y(f)$ was fitted with Eq. 1 and resolved into exponential processes (A), (B), and (C).

$$Y(f) = H + \underset{\text{Process (A)}}{A/(1 + a/fi)} - \underset{\text{Process (B)}}{B/(1 + b/fi)} + \underset{\text{Process (C)}}{C/(1 + c/fi)}. \quad (1)$$

In Eq. 1, f represent frequency of length oscillation, $i = \sqrt{-1}$; a, b, c represent characteristic frequencies of processes (A), (B), (C), respectively, and $2\pi a$, $2\pi b$, $2\pi c$ represent their apparent rate constants; A, B, and C represent respective magnitudes and H is a constant. Process (B) is known as "oscillatory work" and is equivalent to "delayed tension" or "phase 3" in step analysis. Process (C) is equivalent to "phase 2" which was characterized and modeled by Huxley (1974). The details of the sinusoidal analysis technique were previously published (Kawai and Brandt, 1980).

RESULTS

Fig. 1 *A* shows the Nyquist plot obtained from a single fiber preparation which was not treated with EDC, and were activated in a standard solution in the presence of saturating concentrations of Ca and MgATP. Fig. 1 *B* is the result of fitting the data of Fig. 1 *A* with Eq. 1. Note that three processes (A), (B), (C) are readily identified in the plot.

A different fiber was treated with EDC to covalently cross-link myosin heads to actin after the rigor state was induced. To assess the degree of cross-linking, stiffness at high ionic strength (500 mM) relaxing solution was compared with that at high ionic strength rigor solution (see Methods). Based on this analysis, we estimated that 18% of the cross-bridges were covalently cross-linked to actin.

The cross-linked fiber immersed in a high ionic strength relaxing solution was then stretched to varying degrees and complex modulus data were collected. The resulting Nyquist plots which are dramatically different from that of untreated preparations are shown in Fig. 2 *A*. Only processes (B) and (C) are identifiable in the high ionic strength solution, with two low frequency points (0.25, 0.5 Hz) diverging into the fourth quadrant (see branches extending to the right of filled squares in Fig. 2 *A*). The behavior of these low frequency points has not yet been characterized. The magnitude of processes (B) and (C) increased as the cross-linked fibers were stretched, reaching the maximum at a stretch yielding the stress of 44 KN/m^2, and declining again for further stretch. The characteristic frequencies of both processes decreased with stretch (Fig. 2 *A*; observe clockwise rotation of decade frequency points). It is also noticeable that there is some separation of the plots from the origin along the elastic axis, indicating the existence of cross-bridges which do not participate in the production of oscillatory power. In fitting the complex modulus data, process (A)

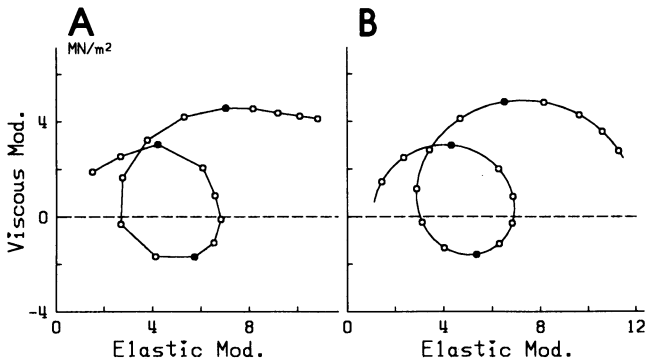

FIGURE 1 Nyquist plot of a normal rabbit psoas fiber activated in physiological ionic strength (200 mM) solution, which contains (mM:) 6 CaEGTA (pCa 4.86), 5.3 MgATP, 4.7 ATP, 8 Pi, 15 CP, 32 KProp, 26 NaProp, 10 MOPS (pH 7.00), 160 U/ml CK. Elastic modulus is plotted on the absicissa, and viscous modulus on the ordinate. Frequencies used are (*clockwise*) 0.25, 0.5, 1, 2, 3.1, 5, 7.5, 11, 17, 25, 35, 50, 70, 100, 135, 186, 250, 350 Hz. Decade frequencies (1, 11, 100 Hz) are shown in filled symbols. Part *A* is based on the actual data, part *B* is the best fit of the data with Eq. 1.

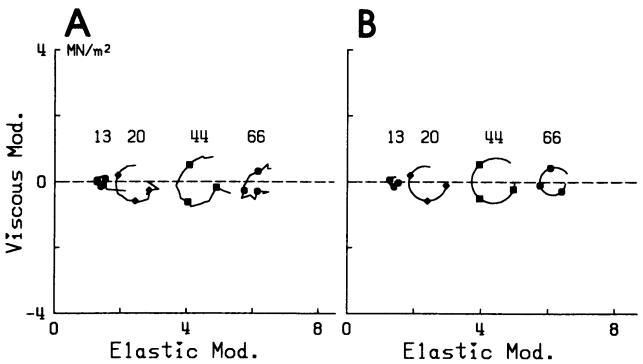

FIGURE 2 Nyquist plots of an EDC cross-linked fiber in high ionic strength (500 mM) solution containing (in millimolars) 6 EGTA, 5.3 MgATP, 4.7 ATP, 8 Pi, 15 CP, 32 KProp, 26 NaProp, 300 KCl, 10 MOPS (pH 7.00), 160 U/ml CK. Filled symbols identify decade frequencies (1, 11, 100 Hz). The stress on the fiber are shown in KN/m^2 in the corresponding plot. *A* is based on the actual data; *B* is the best fit of the data with Eq. 1.

was eliminated from Eq. 1, and frequency points at 0.25 and 0.5 Hz were excluded. The corresponding complex modulus data calculated by Eq. 1 are shown in Fig. 2 *B*. In Fig. 2 *B*, the clockwise rotation of the frequency points with increasing stress is readily seen. In separate experiments, cross-linked fibers were tested in 1,000 mM ionic strength solution with the identical results. The above results were not modified when CaEGTA replaced EGTA, indicating the absence of Ca sensitivity in the cross-linked preparation.

The same preparation was activated in a physiological ionic strength (200 mM) solution which contained saturating Ca^{2+} and MgATP concentrations. The complex modulus data were again collected at varying degrees of stretch. Once again, the complex modulus data (Fig. 3 *A*) are substantially different from conventional Nyquist plot of rabbit psoas fibers (Fig. 1), and they are reminiscent of insect flight muscles (Machin and Pringle, 1960; Rüegg and Tregear, 1966; Pringle 1967; Thorson and White, 1969, 1984; Abbott, 1973). The complex modulus data are characterized by large amounts of oscillatory work, with the low and medium frequency points going through an arc in the fourth quadrant, which we identify as process (B). The high frequency points go through an arc in the first quadrant, which we identify as process (C). Process (A), a low frequency exponential lead and present in untreated fast twitch fibers (Fig. 1), is barely detectable in this cross-linked preparation. Fitting the data with Eq. 1 (shown in Fig. 3 *B*) revealed that the magnitude of parameter *A* was significantly diminished and *H* was increased by the cross-linking, while other parameters (including the rate constants) remained approximately the same with the cross-linking.

It is seen in Fig. 3 that the oscillatory work (process B) increases with stretch to reach the maximum at 88 KN/m^2. It decreases again with further stretch, but this particular preparation was not stretched beyond this point, to avoid damage to the preparation. Note that this optimal stress is greater in the physiological ionic strength solution than in high ionic strength solution. Also note that there is little rotation of individual frequency points indicating that there is little change in rate constants with stretch; this result contrasts with that (Fig. 2) in high ionic strength solution. In experiments with both ionic strength solutions, the effect of stretch is similar to that of insect fibrillar muscles (Abbott, 1973), and cross-linked rabbit psoas fibers exhibit a stretch activation.

As shown in our previous study (Tawada and Kimura, 1986) and others (Takashi, 1988), the result in high ionic strength solution (Fig. 2) reflects the kinetics of covalently cross-linked cross-bridges to actin, whereas that in physiological ionic strength solution (Fig. 3) reflects the kinetics of both cross-linked and noncross-linked cross-bridges (see Discussion). To extract the kinetics of cross-bridges which are noncross-linked to actin, the results of Fig. 2 *A* were vectorially subtracted from the results of Fig. 3 *A* and plotted in Fig. 4 *A*. This subtraction assumes that properties of the cross-linked cross-bridges are not altered by the change in the ionic strength. Because an exact match in stress was not always available from experiments at both ionic strength, the data from the nearest stress was chosen for subtraction. These procedures do not alter the results seriously, because cross-linked cross-bridges contribute much less than noncross-linked cross-bridges under the present condition (compare Fig. 2 vs. Fig. 4). The subtracted results were

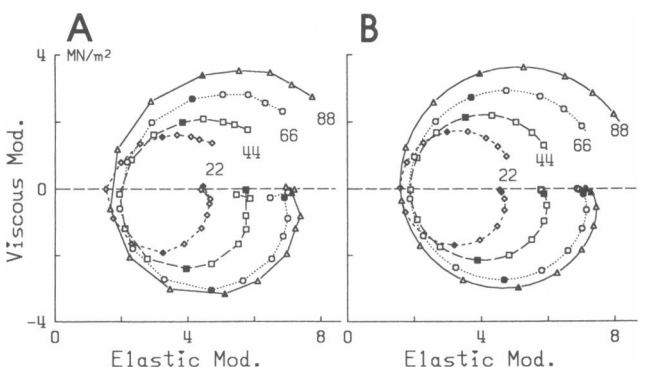

FIGURE 3 Nyquist plots of the EDC cross-linked fiber in physiological ionic strength (200 mM) solution containing (in millimolars) 6 CaEGTA (pCa 4.82), 5.3 MgATP, 4.7 ATP, 8 Pi, 15 CP, 32 KProp, 26 NaProp, 10 MOPS (pH 7.00), 160 U/ml CK. This is the same fiber as that used in Fig. 2; the same plotting convention as in Fig. 1 was used. The stress on the fiber is shown in KN/m^2 in the corresponding plot. *A* is based on the actual data; *B* is the best fit of the data with Eq. 1.

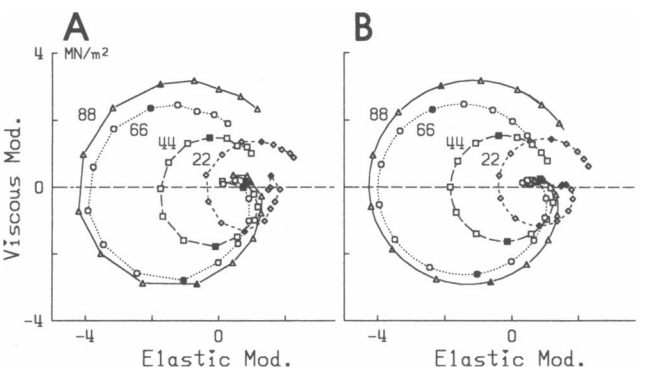

FIGURE 4 Difference Nyquist plots (Fig. 3 minus Fig. 2) to show the complex modulus (frequency response function) of noncross-linked cross-bridges. The strain on the fiber is shown in KN/m^2 in the corresponding plot. Because high ionic strength data at 22 and 88 KN/m^2 were not available, data at 20 and 66 KN/m^2, respectively, were used for subtraction. Part *A* is based on the actual data, part *B* is the best fit of the data with Eq. 1.

fitted with Eq. 1 and shown in Fig. 4 *B*. After the subtraction, fitting of the data with Eq. 1 improved, indicating that the individual exponential process becomes purer with subtraction (compare Fig. 4, *A* and *B*). It is also noticed that characteristic frequencies do not change with imposed stretch, as evidenced by the same relative location of individual frequency points in Nyquist plots.

With EDC treatment, the complex modulus data are much better described by Eq. 1 than without treatment. This is most evident at high frequency. It is likely that another exponential process is needed for satisfactory fit of the data from untreated preparations (Fig. 1), whereas departure of the data (Fig. 4 *A*) from the theoretical projection (Fig. 4 *B*) based on Eq. 1 are much less evident in EDC-treated preparations. From this observation, we infer that EDC-treatment eliminated an undesirable element of the sarcomere which interfered with the sinusoidal analysis.

DISCUSSION

There are several important observations made in the current study. These are (*a*) myosin heads which are covalently cross-linked to actin can produce oscillatory work (Fig. 2) indicating that cross-linked heads are able to catalyze ATP hydrolysis, (*b*) apparent rate constants of cross-linked heads diminish with stretch, (*c*) noncross-linked cross-bridges in an EDC-treated preparation produce significant amounts of oscillatory work (Fig. 4) as a result of diminished magnitude *A*, (*d*) their rate constant remains the same upon stretches, and (*e*) with cross-linking, the complex modulus data become reminiscent of those for insect flight muscles (Machin and Pringle, 1960; Rüegg and Tregear, 1966; Pringle 1967; Thorson and White, 1969, 1984; Abbott, 1973).

There are apparently two kinds of cross-bridges in the EDC-treated, partially cross-linked fibers. One is covalently cross-linked to actin, and the other is not cross-linked to actin and freely mobile. The activity of the cross-linked cross-bridges can be observed in high ionic strength solution (500–1,000 mM). In this condition, noncross-linked cross-bridges can be assumed not to play a role in actin and myosin interaction. These assumptions are supported by observations that, in high ionic strength solutions, actin-activated MgATPase activity is absent if myosin heads are not cross-linked to actin, whereas the MgATPase is superactive when myosin heads are cross-linked to actin in isolated protein systems in solution (Mornet et al, 1981; Takashi, 1988; Huang and Tawada, manuscript submitted for publication). It appears that Ca is not needed to activate the cross-linked cross-bridges, reflecting the high local myosin-head concentration due to cross-linking. This phenomenon is probably related to the earlier finding of activity in the absence of Ca at low ATP concentrations (Bremel and Weber, 1972).

It is interesting to note that cross-linked cross-bridges produce oscillatory power (Fig. 2), with MgATP as the energy source. This is in accord with the results obtained from EDC cross-linked acto-S1 complex: (*a*) the complex exhibits high ATPase activity of the actomyosin-type even at ionic strengths as high as 500 mM; (*b*) myosin heads in the complex show ATP-induced structural changes and motions, as revealed by electron microscopy (Craig et al., 1985) and EPR spectroscopy (Svensson and Thomas, 1986), respectively.

Not all cross-linked cross-bridges are able to catalyze energy transduction. In Fig. 2, if one relates the diameter of the arcs in the Nyquist plot to the number of active cross-bridges, their separation from the origin along the elastic axis may relate to the number of inactive cross-bridges that are cross-linked to actin. From Fig. 2, we conclude that perhaps one-third of the cross-linked cross-bridges are active and two-thirds inactive. The inactive cross-bridges were possibly the result of cross-linking a portion of the myosin molecule essential for transduction. If a cross-linked cross-bridge is inactive, it merely adds a parallel elastic element to an active cross-bridge, and results in the rightward shift of the Nyquist plot along the elastic axis as seen in Fig. 2.

The kinetics of mobile cross-bridges in an EDC-treated preparation can be studied under physiological ionic strength conditions (200 mM; Fig. 3). Ca^{2+} was added to ensure full activation, but this might not be necessary in a more highly cross-linked preparation. With our technique, both cross-linked and noncross-linked cross-bridges are observed under physiological ionic strength conditions. For more rigorous analysis, the contribution of the cross-linked cross-bridges was subtracted from the physiological ionic strength experiments (Fig. 4). As evidenced in Fig. 4, all three processes (A), (B), and (C) are present in noncross-linked cross-bridges. Furthermore, the contribution of process (B) (oscillatory work) is enhanced in the noncross-linked cross-bridges. The analysis of the data in Fig. 4 *A* in terms of Eq. 1 reveals that the apparent increase in oscillatory work is the result of decreased magnitude *A* after EDC treatment. By comparing Figs. 1 and 4, it can be concluded that the decrease in *A* is about fivefold, whereas the magnitudes *B* and *C* remain approximately the same. *H* also increases with EDC-treatment by keeping $H + A$ approximately constant. All apparent rate constants are little affected by the treatment.

It is interesting that neither cross-linked cross-bridges

at high ionic strength (Fig. 2), nor noncross-linked mobile cross-bridges in physiological ionic strength (Fig. 4) exhibit appreciable evidence of process (A) (or very small when present). Process (A) is the slowest exponential component normally found in fast twitch fibers such as in rabbit psoas (Fig. 1); process (A) corresponds to "phase 4" of step analysis (Huxley, 1974). Because both types of cross-bridges in the EDC-treated preparation produce oscillatory work, we infer that process (A) is not essential for energy transduction, and it may not represent an elementary step in the cross-bridge cycle. Thus, we conclude that process (A) does not reflect a property of a cross-bridge, but may reflect a larger motion such as filament sliding or sarcomere rearrangement.

If such a motion is absent owing to the stabilization of molecular architecture of the sarcomere, process (A) may disappear. Those elements responsible for stabilization are cross-links either in the rod portion of myosin molecules, or in the contact region between the myosin head and actin molecules. The rod portion of myosin molecules, which is a rigid coiled-coil alpha-helical structure, is known to be covalently cross-linked to thick filament under the cross-linking condition we used (Tawada and Kimura, 1986). Because process (A) is an exponential advance, which consumes mechanical energy to produce heat, its decline results in an increase in the apparent power production against forcing device.

In insect muscles, it is generally believed that rigid sarcomeres and the presence of C-filaments that interconnect thick filaments to the *Z*-line are responsible for the phenomenon of stretch activation and the high degree of oscillatory power production (Thorson and White, 1969, 1984). It is also believed that matching of actin and myosin helical periodicity causes the actin and myosin interaction depend on the sarcomere length (Wray, 1979), although the precise mechanism is yet to be worked out. Our findings, that one can make a rabbit muscle mimic the kinetics of insect muscles by creating cross-links within a sarcomere, may help elucidate the molecular mechanism which leads to oscillatory power production.

This work was supported in part by grants from Itoh Science Foundation of Japan and from the Ministry of Education, Science, and Culture of Japan to K. Tawada; grants from NIH AR 21530 and AHA 88-0729 to M. Kawai. M. Kawai was an Established Investigator of the American Heart Association.

Received for publication 12 June 1989 and in final form 1 November 1989.

REFERENCES

Abbott, R. H. 1973. The effects of fibre length and calcium ion concentration on the dynamic response of glycerol extracted insect fibrillar muscle. *J. Physiol. (Lond.)* 231:195–208.

Bremel, R. D., and A. Weber. 1972. Cooperation within actin filament in vertebrate skeletal muscle. *Nat. New Biol.* 238:97–101.

Craig, R., L. E. Green, and E. Eisenberg. 1985. Structure of the actin-myosin complex in the presence of ATP. *Proc. Natl. Acad. Sci. USA.* 82:3247–3251.

Eisenberg, E., and T. L. Hill. 1985. Muscle contraction and free energy transduction in biological systems. *Science (Wash. DC).* 227:999–1006.

Huxley, A. F. 1974. Muscular contraction. *J. Physiol. (Lond.)* 243:1–43.

Kawai, M., and P. W. Brandt. 1976. Two rigor states in skinned crayfish single muscle fibers. *J. Gen. Physiol.* 68:267–280.

Kawai, M., and P. W. Brandt. 1980. Sinusoidal analysis: a high resolution method for correlating biochemical reactions with physiological processes in activated skeletal muscles of rabbit, frog and crayfish. *J. Muscle Res. Cell Motil.* 1:279–303.

Kawai, M., and H. R. Halvorson. 1989. Role of MgATP and MgADP in the cross-bridge kinetics in chemically skinned rabbit psoas fibers. Study of a fast exponential process (*C*). *Biophys. J.* 55:595–603.

Kawai, M., and K. Tawada. 1990. Covalent cross-linking of single muscle fibers increases the oscillatory power production, exhibiting an insect response. *Biophys. J.* 57:410*a*. (Abstr.)

Machin, K. E., and J. W. S. Pringle. 1960. The physiology of insect fibrillar muscle. III. The effect of sinusoidal changes of length on a beetle flight muscle. *Proc. R. Soc. Lond. B Biol. Sci.* 152:311–330.

Mornet, D., R. Bertrand, P. Pantel, E. Audemard, and R. Kassab. 1981. Structure of the actin-myosin interface. *Nature (Lond.).* 292:301–306.

Pringle, J. W. S. 1967. The contractile mechanism of insect fibrillar muscle. *Prog. Biophys. Mol. Biol.* 17:1–60.

Rüegg, J. C., and R. T. Tregear. 1966. Mechanical factors affecting the ATPase activity of glycerol-extracted fibres of insect fibrillar muscle. *Proc. R. Soc. Lond. B Biol. Sci.* 165:497–512.

Svensson, E. C., and D. D. Thomas. 1986. ATP induces microsecond rotational motions of myosin heads cross-linked to actin. *Biophys. J.* 50:999–1002.

Takashi, R. 1988. A novel actin label: a fluorescent probe at glutamine-41 and its consequences. *Biochemistry.* 27:938–943.

Tawada, K., and M. Kimura. 1986. Stiffness of carbodiimide-crosslinked glycerinated muscle fibres in rigor and relaxing solutions at high salt concentrations. *J. Muscle Res. Cell Motil.* 7:339–350.

Thorson, J., and D. C. S. White. 1969. Distributed presentations for actin-myosin interaction in the oscillatory contraction of muscle. *Biophys. J.* 9:360–390.

Thorson, J., and D. C. S. White. 1984. Role of cross-bridge distortion in the small-signal mechanical dynamics of insect and rabbit striated muscle. *J. Physiol. (Lond.).* 343:59–84.

Wray, J. S. 1979. Filament geometry and the activation of insect flight muscles. *Nature (Lond.).* 280:325–326.

Yanagida, T., T. Arata, and F. Oosawa. 1985. Sliding distance of actin filament induced by a myosin cross-bridge during one ATP hydrolysis cycle, *Nature (Lond.).* 316:366–369.