Schroeder, W. A. (1967), *Methods Enzymol. 11*, 351, 361.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6*, 3315.
Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5*, 2108.
Somogyi, M. (1957), *Methods Enzymol. 3*, 3.
Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 29*, 1249.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci.* (in press).
Velick, S. F., and Wicks, L. F. (1951), *J. Biol. Chem. 190*, 741.
Wada, H., and Snell, E. E. (1961), *J. Biol. Chem. 236*, 2089.
Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.
Wang, J. H., and Graves, D. J. (1963), *J. Biol. Chem. 238*, 7.
Wang, J. H., and Graves, D. J. (1964), *Biochemistry 3*, 1437.
Wang, J. H., Shonka, M. L., and Graves, D. J. (1965), *Biochemistry 4*, 2296.
Wolf, D. P. (1967), Ph.D. Thesis, University of Washington, Seattle, Wash.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Conformational Changes in the Molecular Control of Muscle Contraction*

Yuji Tonomura,† Shizuo Watanabe, and Manuel Morales‡

ABSTRACT: The McConnell "spin-label" technique (mainly SH-directed labels) has been used to examine whether a structural effect is transmitted across the protein system which controls the contraction of muscle: troponin–tropomyosin–actin. It was found that a spin label attached to tropomyosin responds to Ca^{2+} fluctuations in the micromolar range only when troponin is present, and that a spin label attached to actin responds only when troponin–tropomyosin are present. Thus some structural effect appears to be communicated to actin across this control system.

The contractile system of muscle is thought to be controlled by changes in intrafibrial [Ca^{2+}], and this control to be exerted through tropomyosin and troponin, proteins which are attached probably at regular intervals to the F-actin of the thin filaments. In the absence of Ca^{2+} these attachments prevent the force-generating interactions between F-actin and the myosin of the thick filaments. Approximately micromolar Ca^{2+} is thought to nullify this prevention, thus restoring force generation; in this sense Ca^{2+} is said to "activate" muscle. Since the tropomyosin–F-actin complex is stable and the troponin–F-actin complex is not, and since troponin–tropomyosin is stable, it is thought that the control arrangement is troponin–tropomyosin–F-actin. Moreover, Ca^{2+} has a high affinity for troponin, or for the mixture troponin–tropomyosin, a mixture which when unresolved has been called "relaxing protein," but myosin reacts directly with F-actin; therefore, it seems that there is some form of communication between one end and the other of Ca^{2+}–troponin–tropomyosin–F-actin–myosin. The foregoing ideas are largely the fruit of elegant work by Ebashi and his associates (Ebashi and Ebashi, 1964; Ebashi and Kodama, 1966; Endo *et al.*, 1966; Ebashi *et al.*, 1967; Ohtsuki *et al.*, 1967). In this paper we examine whether some effect is indeed transmitted across the system troponin–tropomyosin–F-actin when [Ca^{2+}] is varied in the micromolar range. For this purpose we employed the "spin-label" technique of McConnell and coworkers (Hamilton and McConnell, 1968), *i.e.*, the attaching to the protein under study of stable, environment-sensing free radicals whose electron paramagnetic resonance spectrum can be followed as the labeled protein participates in its reactions. Mainly we employed spin labels directed to the SH groups of the proteins; of the two employed, the analog of *N*-ethylmaleimide was more useful than the analog of iodoacetamide. In one instance we used a spin label directed to NH_2 groups, but perhaps also reactive toward SH groups, *viz.*, the analog of isothiocyanate. Our strategy of labeling was to label in

* From the Cardiovascular Research Institute, University of California, San Francisco, California 94122. *Received December 2, 1968.* Research supported by Grant GB 7531 from the National Science Foundation, Grant C.I.8 from the American Heart Association, and Grant GM 14076 from the National Institute of General Medical Sciences.

† Visiting Scientist Awardee, American Heart Association. On leave from the Department of Biology, Faculty of Science, University of Osaka.

‡ Career Investigator, American Heart Association.

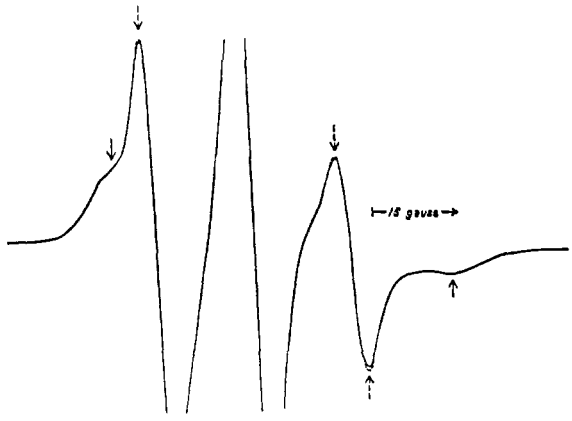

FIGURE 1: Effect of Ca^{2+} on electron paramagnetic resonance spectrum of X-troponin. Troponin was incubated with 3 moles of X/10^5 g for 66 hr; 1.46 mg/ml X-troponin, 0.058 M KCl, 1.5 mM $MgCl_2$, and 20 mM Tris-maleate (pH 7.0) at room temperature. (——) 0.2 mM EGTA; (- - - -) 0.1 mM $CaCl_2$. In all figures on electron paramagnetic resonance spectrum, (↓) and (⇣) indicate a strongly immobilized signal and a weakly immobilized one, respectively.

such a way that the spectra revealed changes when the protein participated in reactions of interest, but also in such a way that the label did not impede these reactions.

Experimental Section

Preparations. F-actin and myosin were prepared according to Mommaerts (1952), with slight modifications, and Tonomura *et al.* (1966), respectively. Tropomyosin, troponin, relaxing protein, and EGTA-desensitized myosin B (*i.e.*, F-actin–myosin largely freed of relaxing protein) were prepared according to Azuma and Watanabe (1965), Arai and Watanabe (1968),[1] Watanabe and Staprans (1966),[1] and Perry *et al.* (1966), respectively. Following preparation, F-actin and relaxing protein were dialyzed against 30 mM KCl plus 5 mM Tris-HCl, pH 8.0, 4°, for 1–1.5 days, then allowed to react in the same solvent for 1–1.5 days (initial reagent/protein ratios are given in figure legends); troponin was reacted with the analog of *N*-ethylmaleimide in neutral, 4°, in 0.2 mM $KHCO_3$ for 2.5 days; troponin was reacted with the analog of isothiocyanate in neutral, 4°, in 2 mM $KHCO_3$ for 1 day. Finally, all solutions were dialyzed against 30 mM KCl plus 5 mM Tris-maleate, pH 7.0, 4°, for a day or more.

N-2,2,6,6-Tetramethylpiperidine nitroxide, maleimide (X), and *N*-2,2,6,6-tetramethylpiperidine nitroxide, isothiocyanate (Y), were purchased from Varian Associates, Calif. *N*-2,2,6,6-Tetramethylpiperidine nitroxide, iodoacetamide (Z), was prepared by S. Clift and J. A. Duke, according to instructions kindly provided by Professor H. M. McConnell.

Electron Paramagnetic Resonance Spectra. Spectra were obtained with a Varian Associates type E-3 spectrometer. The field strength was set at 3380 G, with a scan range of ±50 G. During scanning the time constant of detection was 0.3 and 1.0 sec, and the scan time was 4 and 8 min. The modulation frequency was 100 kHz, and the amplitude, 6.3 G. The microwave frequency was 9.515–9.520 GHz, and the power 100 mW. The receiver gain was 2×10^4 to 5×10^5. Generally, two measures were made at high gain and two at low gain.

The solvent (all components except the labeled protein) was kept at room temperature. Cold labeled protein was added to solvent, mixed, kept at room temperature for 8 min, then put into a special glass liquid sample cell supplied by Varian Associates and measured.

Occasionally, time-dependent spectral changes were observed due to microwave heating effects. Such circumstances were carefully avoided, and in later experiments cooling air was circulated through the cavity. All spectra reported here were taken under stable conditions, and reproducibility of reported spectra was good enough so that successive spectra could be superimposed one on the other to average out noise. Since size of signal depends upon cell location in the cavity, spectra to be compared were normalized with respect to the difference between the peak at 3372.5 G and the trough at 3378.5 G.

The spectra of nitroxide type labels have been extensively discussed by Hamilton and McConnell (1968). Since all labeled proteins we used were exhaustively dialyzed after labeling, the observed spectra can be assumed to arise from labels which are covalently bound to the protein; however, some of these labels enjoy unrestricted motion *relative* to the protein and others do not. Peaks at ~3357 and ~3390 G and the trough at ~3395 G arise from what McConnell terms "weakly immobilized" (here called "loose") labels and peak at ~3350 G and the trough at ~3413 G arise from what he terms "strongly immobilized" (here called "tight") labels. The viscosity increase resulting from adding sucrose to the system was often used to identify the two classes of label. In what follows we refer to "increases" and "decreases" in peaks arising from loose or tight labels; it must be remembered that these manifestations are only indirectly related to numbers of labels in one state or another, since the ordinate in electron paramagnetic resonance spectra is the *derivative* of energy absorbed with respect to field.

Other Measurements. Functional assay of the control system (troponin + tropomyosin, or relaxing protein) consisted in judging its effect on clearing and superprecipitation of actomyosin, as observed at 550 mμ with a recording photometer. Kinetics of dissolved actomyosin formation or dissociation (by ATP) were recorded as changes in transmission at 400 mμ, using a modified Gibson-Durrum stopped-flow apparatus.

Buffering [Ca^{2+}]. For purposes of calculation, 4.8×10^4 was taken as the molecular weight of actin monomer, and 2×10^{-7} M as the dissociation constant, in 0.05–0.06 M KCl, at pH 7.0, 25°, of the complex of Ca^{2+} and EGTA, *i.e.*, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetate.

[1] Purity of the relaxing proteins was of the best yet reported; characterization is given by Arai and Watanabe (1968) and by Watanabe and Staprans (1966).

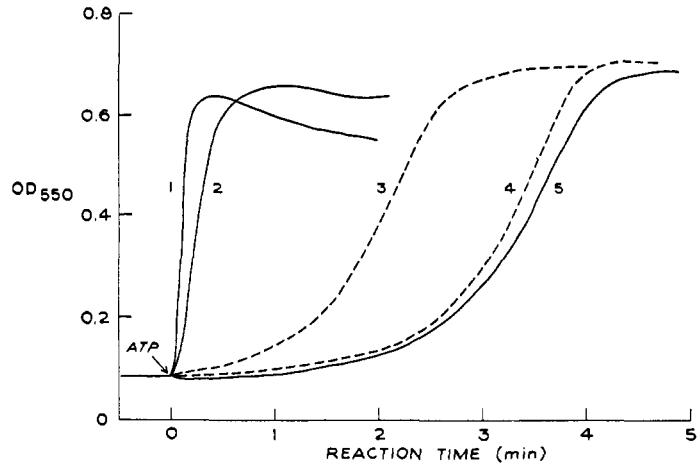

FIGURE 2: Effect of modifications of troponin by Y and tropomyosin by X on their physiological functions; 0.2 mg/ml of densensitized myosin B, 0.04 M KCl, 0.2 mM EGTA, 0.2 mM $MgCl_2$, 0.2 mM ATP, and 20 mM Tris-maleate, pH 6.9, 25°. Y-troponin was obtained by incubation with 2 moles of Y/10^5 g for 21 hr. X-tropomyosin was obtained by incubation of 1.45 moles of X/10^5 g for 2 days.

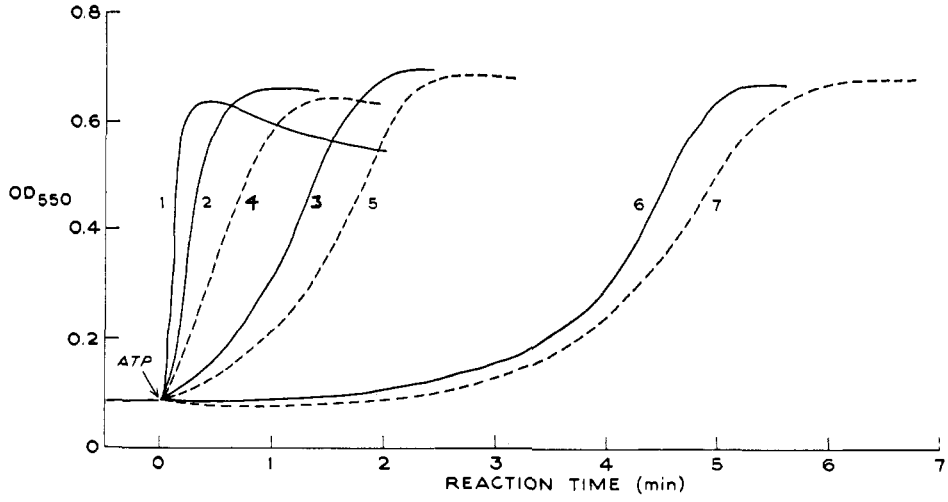

FIGURE 3: Effect of modification of relaxing protein by X on its relaxing activity; 0.2 mg/ml of desensitized myosin B, 0.04 M KCl, 0.2 mM EGTA, 0.2 mM $MgCl_2$, 0.2 mM ATP, and 20 mM Tris-maleate, pH 6.9, 25°. X-tropomyosin as used in Figure 2. X-relaxing protein was obtained by incubation with 1.27 moles of X/10^5 g for 2 days. ① control; ② +25 μg/ml of tropomyosin; ③ +50 μg/ml of tropomyosin; ④ +38 μg/ml of X-tropomyosin; ⑤ +80 μg/ml of X-tropomyosin; ⑥ +45 μg/ml of relaxing protein; ⑦ +45 μg/ml of X-relaxing protein. Dotted curves refer to labeled proteins.

Results

When X is attached to troponin the resulting spectrum shows large signals from loose labels and small signals from tight labels (Figure 1). Although Ca^{2+} has a high affinity for troponin (Ebashi *et al.*, 1967; Arai and Watanabe, 1968), it has no effect on the spectrum of X-troponin (Figure 1), or of X-troponin-tropomyosin. Reaction of troponin with X to the extent required for labeling has almost no effect on its function. When Y is attached to troponin the resulting spectrum shows only signals from loose labels, and it is insensitive to Ca^{2+} addition, even in the presence of tropomyosin; moreover, Y-troponin-tropomyosin is functionally ineffective to some extent (Figure 2). It may be that the SH groups of troponin, though probably nonparticipants in its function (*cf.* Arai and Watanabe, 1968; Staprans *et al.*, 1968), are remote from the region in which Ca^{2+} binds or conformation changes; the Y results, on the other hand, suggest that one or more of the NH_2 groups of troponin participates in its function.

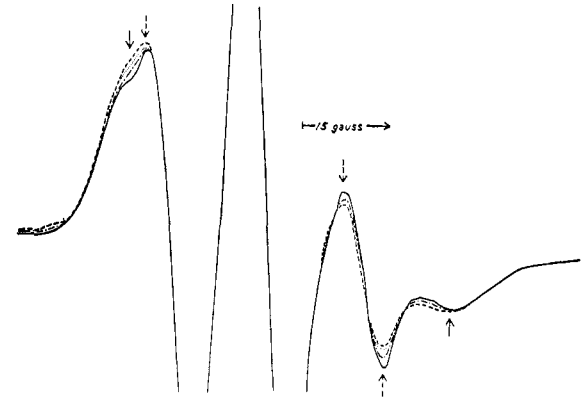

FIGURE 4: Effect of Ca^{2+} on electron paramagnetic resonance spectrum of X-relaxing protein. Relaxing protein was incubated with 1.42 moles of X/10^5 g for 54 hr, 4 mg/ml of X-relaxing protein, 0.054 M KCl, 1.5 mM $MgCl_2$, and 20 mM Tris-maleate, pH 7.0, at room temperature. (——) 0.3 mM EGTA; (—·—) 0.1 mM $CaCl_2$ plus 0.3 mM EGTA (free Ca^{2+}, 0.1 μM); (· · · ·) 0.1 mM $CaCl_2$ plus 0.17 mM EGTA (free Ca^{2+}, 0.3 μM); (– – –) 0.1 mM $CaCl_2$.

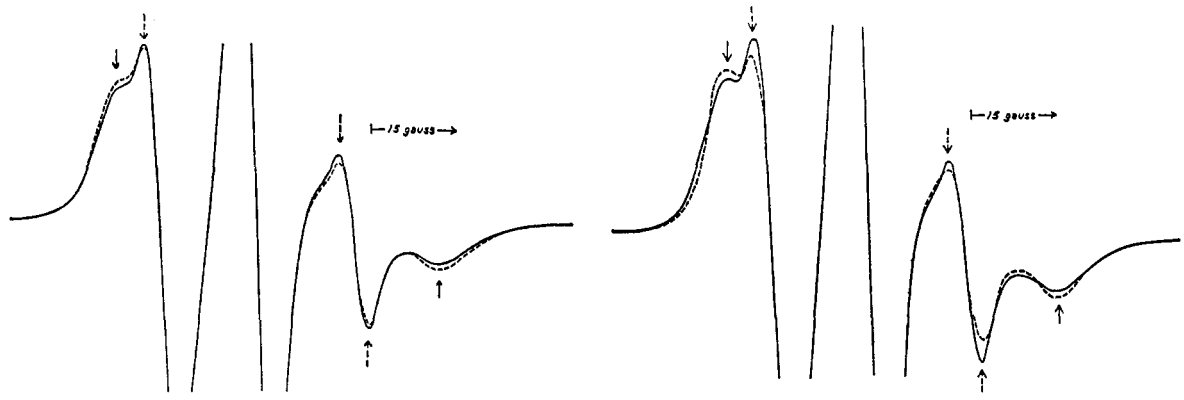

FIGURE 5: Effect of Ca^{2+} on electron paramagnetic resonance spectrum of X-relaxing protein in the absence and the presence of F-actin. Relaxing protein was incubated with 1.64 moles of X/10^5 g for 3 days; 0.07 M KCl, 1.5 mM $MgCl_2$, and 20 mM Tris-maleate, pH 7.0, at room temperature. (A) Left: 2.87 mg/ml of X-relaxing protein; (—) 0.2 mM EGTA; (- - -) 0.1 mM $CaCl_2$. (B) Right: 1.55 mg/ml of X-relaxing protein plus 1.9 mg/ml of F-actin; (——) 0.2 mM EGTA; (- - -) 0.1 0.1 mM $CaCl_2$.

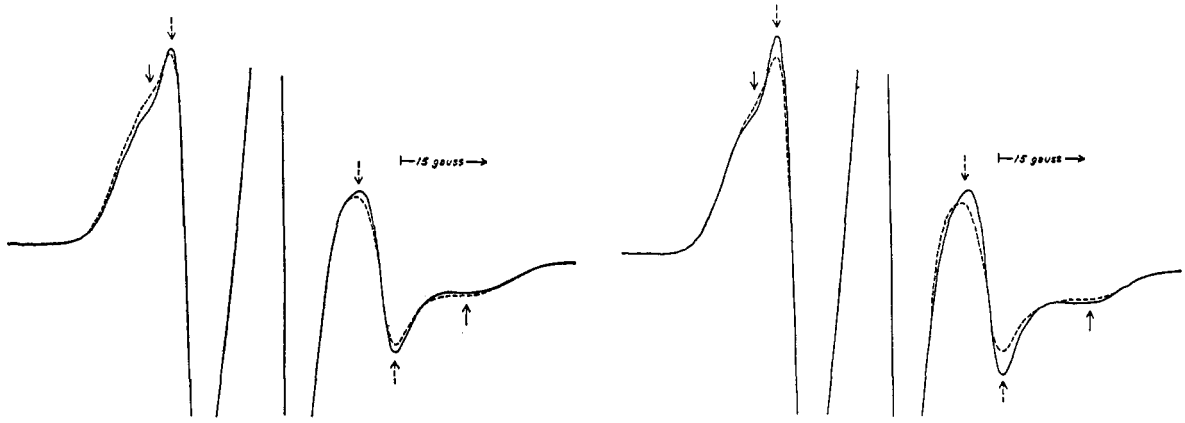

FIGURE 6: Effect of Ca^{2+} on electron paramagnetic resonance spectrum of X-tropomyosin-troponin in the absence and the presence of F-actin. Tropomyosin was incubated with 1.45 moles of X/10^5 g for 2 days. (A) Left: 2.34 mg/ml of X-tropomyosin, 1.19 mg/ml of troponin, 0.058 M KCl, 1.5 mM $MgCl_2$, and 20 mM Tris-maleate, pH 7.0. (——) 0.2 mM EGTA; (- - - -) 0.1 mM $CaCl_2$. (B) Right: 1.87 mg/ml of X-tropomyosin, 0.6 mg/ml of troponin, 1.51 mg/ml of F-actin, 0.064 M KCl, 1.5 mM $MgCl_2$, and 20 mM Tris-maleate, pH 7.0. (——) 0.2 mM EGTA; (- - - -) 0.1 mM $CaCl_2$.

Staprans *et al.* (1968) have already shown that SH reagents do not impede the function of the relaxing protein system, so both X-relaxing protein and Z-relaxing protein were prepared; however, only the spectrum of the former shows signals from tight as well as from loose labels. Figure 3 shows that X-relaxing protein is functionally effective. The two types of signals observed in the spectrum of X-relaxing protein (Figure 4) are confirmed by the sucrose test. In the presence of a Ca^{2+}-chelator, EGTA, the addition of Mg^{2+} and its removal by EDTA have no effect on the spectrum. On the other hand, when a Ca^{2+}-buffer system is used to impose [Ca^{2+}] concentrations of 0, 1×10^{-7}, 3×10^{-7}, and 10^{-4} M the spectra show a progressive increase in signals from tight labels and a progressive decrease in signals from loose labels (Figure 4). Significantly, the concentration for half-effect is about 10^{-7}, more or less what is deduced from functional testing. Our experience with spectra from several relaxing protein preparations was that while the Ca^{2+} effect was invariably present, its magnitude varied from one preparation to another; perhaps variability in free SH groups directs the labels to various positions. The Ca^{2+} effect just described was not affected by increasing KCl concentration from 0.07 to 0.52 M or by adding myosin to the relaxing protein system; on the other hand, the Ca^{2+} effect is greater on X-relaxing protein–F-actin than on X-relaxing protein (Figure 5A,B).

Absence of a Ca^{2+} effect on spectra from troponin and presence of such an effect on spectra from relaxing protein suggest that an effect might be found on the spectrum of X-tropomyosin. Figure 2 shows that X-tropomyosin functions about as well as tropomyosin; this same figure also suggests that tropomyosin is somewhat contaminated with troponin, since it does have a superprecipitation-suppressing effect, Ca^{2+} has only a slight effect on the spectrum of X-tropomyosin. On the other hand, signals from tight labels are much enhanced when tropomyosin complexes with troponin (system troponin-tropomyosin-X) (Figure 6A), and even more when F-actin adds to the complex (system: troponin-tropomyosin-X-F-actin) (Figure 6B). It seems very likely that the slight Ca^{2+} effect on tropomyosin "alone" is due to its contamination with troponin.

Actin was labeled with X rather than Z because only

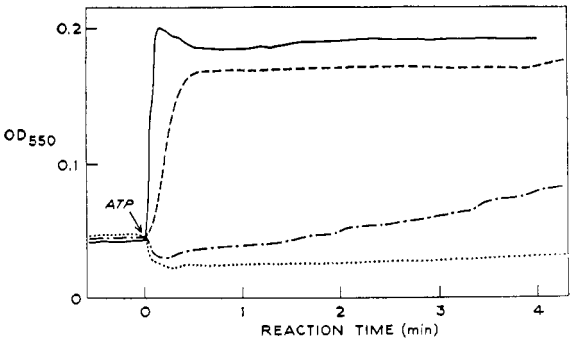

FIGURE 7: Effect of modification of F-actin by *N*-ethylmaleimide on superprecipitation of myosin–F-actin–relaxing protein complex; 0.025 mg/ml of F-actin, 0.075 mg/ml of myosin, 0.02 mg/ml of relaxing protein, 0.06 M KCl, 1 mM $MgCl_2$, and 20 mM Tris-maleate, pH 7.0, 25°. (——) No *N*-ethylmaleimide, 20 μM $CaCl_2$; (·—·—·) no *N*-ethylmaleimide, 0.18 mM EGTA; (– – – –) 1.3 moles of *N*-ethylmaleimide/4.8 × 10^4 g of actin, 20 μM $CaCl_2$; (· · · ·) 1.3 moles of *N*-ethylmaleimide/4.8 × 10^4 g of actin, and 0.18 mM EGTA.

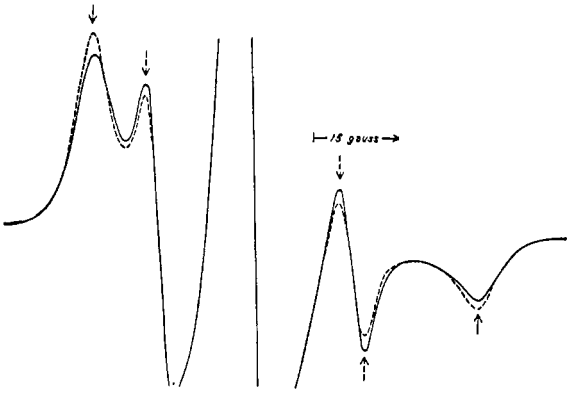

FIGURE 8: Effect of Ca^{2+} on electron paramagnetic resonance spectrum of X–F-actin in the presence of relaxing protein. F-actin was incubated with 1.7 moles of X/4.8 × 10^4 g of actin for 2 days; 1.85 mg/ml of X–F-actin, 0.92 mg/ml of relaxing protein, 0.059 M KCl, 1.5 mM $MgCl_2$, 1.5 mM ATP, and 20 mM Tris-maleate, pH 7.0, at room temperature. (——) 0.24 mM EGTA; (– – –) 0.2 mM $CaCl_2$ plus 0.04 mM EGTA.

the spectrum of F-actin–X showed signals from tight as well as loose labels. Reaction of F-actin with *N*-ethylmaleimide reduces only slightly the superprecipitation and "relaxation" of a relaxing protein, F-actin, and myosin system (Figure 7). Using Boyer's (1954) *p*-mercuribenzoic acid SH titration method with F-actin, it could be shown that all *N*-ethylmaleimide added binds to SH groups. Furthermore, if X is attached to F-actin, or to F-actin previously reacted with *N*-ethylmaleimide, the qualitative features of the spectra are identical; only the amplitudes of the latter are less; therefore it follows that X attaches with the same specificity as *N*-ethylmaleimide. In work with labeled actin care was taken to avoid the effects which slow depolymerization might cause. Ca^{2+} addition to relaxing protein–F-actin–X clearly enhances signals from tight labels and reduces signals from loose labels (Figure 8). In this experiment a reaction medium containing MgATP was used to mimic the physiological one. But MgATP was not essential to the change. Ca^{2+} has a similar but much weaker effect on actin "alone" possibly because of slight relaxing protein contamination.

As expressed earlier, it is thought that the relaxing protein–F-actin interaction in the absence of Ca^{2+} suppresses the F-actin–myosin interaction, and that micromolar concentrations of free Ca^{2+} remove this suppression. Further pursuit of our work would suggest such experiments as adding Ca^{2+} to relaxing protein–F-actin–X–myosin, or to relaxing protein–F-actin–myosin–X; however, addition of a high proportion of myosin to the systems we have studied in [KCl] ~ 0.10 results in technical complications insurmountable at this time. We therefore considered studying such systems in the dissolved state. Since there is little or no information on systems in [KCl] ~ 0.6, we examined the effects of micromolar Ca^{2+} and relaxing protein on the rate of combination of actin and myosin, and on the rate of ATP dissociation of reconstituted actomyosin following the light transmission property in a stopped-flow apparatus. The rate of binding of myosin to F-actin in 0.6 M KCl was independent of Ca^{2+} and Mg^{2+} (Figure 9). This was true even in the presence of relaxing protein. The rate of dissociation of reconstituted actomyosin (myosin and room temperature extracted F-actin, *i.e.*, F-actin plus relaxing protein) by ATP in 0.6 M KCl–1 mM $MgCl_2$ was independent of Ca^{2+} (Figure 10). The rate of recovery phase was also affected very little by Ca^{2+}.

Conclusion

If we assume that (1) troponin, and not tropomyosin, has an affinity for Ca^{2+} ions at micromolar concentra-

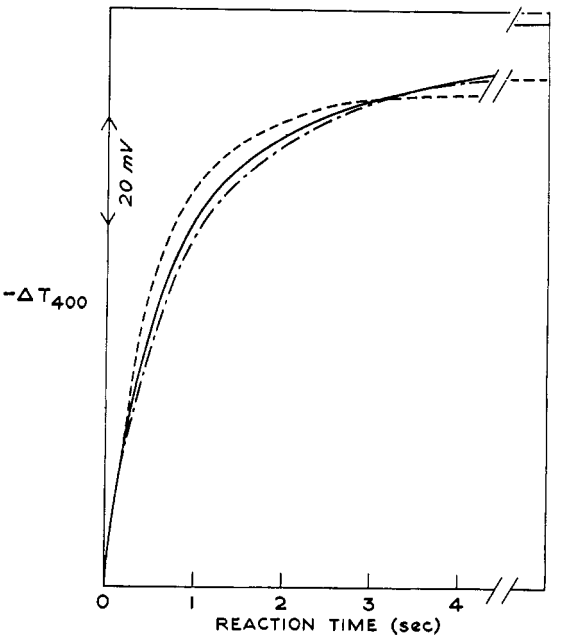

FIGURE 9: Effect of divalent cation on the binding of myosin to F-actin; 0.96 mg/ml of myosin, 0.19 mg/ml of F-actin, 0.6 M KCl, and 20 mM Tris-maleate, pH 7.0, 26°. (——) 1 mM $MgCl_2$ plus 20 μM $CaCl_2$; (—·—·) 1 mM $MgCl_2$ plus 0.18 mM EGTA; (– – – –) 0.2 mM EDTA.

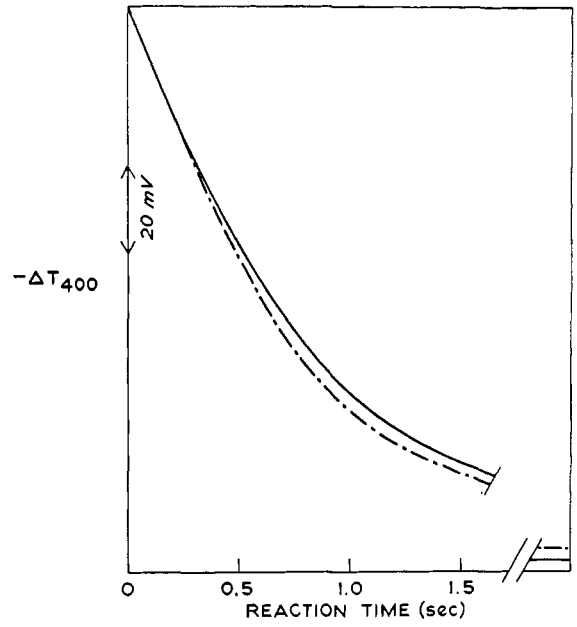

FIGURE 10: Effect of Ca^{2+} on dissociation of reconstituted actomyosin induced by ATP; 0.96 mg/ml of myosin, 0.19 mg/ml of room temperature extracted F-actin, 0.6 M KCl, and 20 mM Tris-maleate, pH 7.0, 26°. (——) 1 mM $MgCl_2$ plus 4 μM $CaCl_2$; (—·—·) 1 mM $MgCl_2$ plus 0.1 mM EGTA.

tion ranges and (2) a change in the electron paramagnetic resonance spectrum of spin-labeled protein indicates a change in the label environment which is most likely structural or conformational, then the foregoing results support a very simple hypothesis of how control by Ca^{2+} ions is exerted: (a) Ca^{2+} attaches to troponin, inducing a structural change which is transmitted to the tropomyosin with which troponin is complexed; this follows because labels attached to tropomyosin sense an effect when Ca^{2+} binds to troponin. Labels attached to troponin itself do not sense this event, probably because they are disadvantageously situated. (b) The structural change suffered by tropomyosin is transmitted to the F-actin with which tropomyosin is complexed; the structural state of F-actin determines whether or not it will interact with myosin to generate force; this follows because labels attached to F-actin sense the change in relaxing protein, but sense nothing, or very little, in the absence of relaxing protein. (c) There is a mechanical reciprocity between tropomyosin and F-actin; the change in F-actin is enhanced by tropomyosin, but also the change in tropomyosin is enhanced by F-actin. Collectively the foregoing results constitute a substantiation of Ebashi's ideas, at the molecular level.

Acknowledgment

We are very grateful to Dr. Roger Cooke for valuable advice on this work. Miss L. Stowring and Miss S. Clift made several of the preparations.

References

Arai, K., and Watanabe, S. (1968), *J. Biol. Chem. 243*, 5670.

Azuma, N., and Watanabe, S. (1965), *J. Biol. Chem. 240*, 3847.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.

Ebashi, S., and Ebashi, F. (1964), *J. Biochem. (Tokyo) 55*, 604.

Ebashi, S., Ebashi, F., and Kodama, A. (1967), *J. Biochem. (Tokyo) 62*, 137.

Ebashi, S., and Kodama, A. (1966), *J. Biochem. (Tokyo) 59*, 425.

Endo, M., Nomura, Y., Masaki, T., Ohtsuki, I., and Ebashi, S. (1966), *J. Biochem. (Tokyo) 60*, 605.

Hamilton, C. L., and McConnell, H. M. (1968), *in* Structural Chemistry and Molecular Biology, Rich, A., and Davidson, N., Ed., San Francisco, Freeman, Calif., p 15.

Mommaerts, W. F. H. M. (1952), *J. Biol. Chem. 198*, 445.

Ohtsuki, I., Masaki, T., Nomura, Y., and Ebashi, S. (1967), *J. Biochem. (Tokyo) 61*, 817.

Perry, S. V., Davis, V., and Hayter, D. (1966), *Biochem. J. 99*, 1c.

Staprans, I., Arai, K., and Watanabe, S. (1968), *J. Biochem. (Tokyo) 64*, 65.

Tonomura, Y., Appel, P., and Morales, M. (1966), *Biochemistry 5*, 515.

Watanabe, S., and Staprans, I. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 572.