The Contractile and Control Sites of Natural Actomyosin

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ABSTRACT The various contractile and control sites of natural actomyosin gel were studied by comparing the kinetics of ATP hydrolysis with those of gel contraction, measured as an increase in turbidity. Contraction of actomyosin gel seems to require the cooperative reaction of ATP (with Mg) at two different sites. One of these sites catalyzes the hydrolysis of ATP and most probably contributes the driving force for contraction; the binding of ATP to the other site appears to break certain links that retard movement of the gel components. At limiting concentrations of ATP, the rate of contraction seems to depend on the rate of breaking these links as well as on the rate of ATP hydrolysis. But when both sites are saturated, the rate of contraction appears to be limited only by the rate of ATP hydrolysis. In addition to these two contractile sites, there are also two different control sites. At one, the relaxing site, the binding of ATP with Mg inhibits ATP hydrolysis and gel contraction. At the other, the binding of calcium activates contraction by overcoming the inhibitory action of Mg and ATP at the relaxing site. This control system—inhibition by substrate and disinhibition by calcium—can be selectively inactivated by heat and reactivated by dithiothreitol, a disulfide-reducing agent. These observations on the isolated contractile system are discussed in relation to the contraction and relaxation of muscle.

Since the discovery by Szent-Györgyi that natural actomyosin threads contract when exposed to ATP and magnesium ion (3), this isolated contractile complex of muscle proteins has been used in a variety of forms to study the biochemical properties of muscle contraction (4). A particularly useful form for quantitative kinetic studies of contraction is a suspension of small gel particles. When these particles contract, the turbidity of the suspension increases; and the rate of contraction can be measured by recording the increase in optical density (5–7).

Earlier kinetic studies, using this optical method, have indicated that MgATP operates at a number of different sites in the actomyosin system. This paper describes further studies aimed at understanding the properties of

these various contractile and control sites and their function in the contraction and relaxation of muscle proteins.

MATERIALS AND METHODS

Preparation of Natural Actomyosin Gel Stock suspensions of natural actomyosin from rabbit back muscle were prepared exactly as described in a previous paper (7) and stored at 0°C. In this preparation the protein is extracted with Weber-Edsall solution and reprecipitated twice by dilution from 0.6M to 0.1 M KCl. In addition to the main constituents, actin and myosin, the gel most probably contains tropomyosin and small amounts of other proteins that may coprecipitate at 0.1 M KCl.

Measurement of Superprecipitation Superprecipitation of the gel particles in suspension was measured by following the change in turbidity at 545 mμ in a Zeiss spectrophotometer (7); the suspension was stirred continuously with a magnetic stirrer and maintained at constant temperature. The reaction solution contained 0.03 M KCl, 0.06 M Tris, 0.005 M $MgCl_2$, 1×10^{-5} M $CaCl_2$, and 0.12 mg/ml actomyosin, pH 7.4 at each stated temperature. 0.002 M EGTA was added when indicated. In some experiments the solution was buffered with a mixture of Ca and EGTA to maintain a free calcium ion concentration of about 1×10^{-5} M (8). When measuring the rate of superprecipitation at low substrate concentrations, creatine phosphate and creatine phosphotransferase (EC 2.7.3.2) were included in the reaction solution to maintain the ATP concentration.

Measurement of Hydrolysis $AT^{32}P$ hydrolysis was determined by measuring the $^{32}P_i$ released in 10 sec. The conditions were the same as those for measuring superprecipitation. The protein concentration was adjusted so that no more than 10% of the substrate was hydrolyzed during the 10 sec reaction period. Hydrolysis was stopped with trichloroacetic acid, 1 μmole of unlabeled carrier inorganic phosphate added, and the total P_i separated using the isobutanol extraction method (9). An aliquot of the alcohol phase was added to phosphor and the radioactivity was measured in a liquid scintillation counter. K_m and V_{max} values were calculated from Lineweaver-Burk plots.

Heat Inactivation of the Relaxing Site A reaction solution containing 0.03 M KCl, 0.005 M $MgCl_2$, 0.06 M Tris, pH 7.4 at 25°C, was heated to 43°C. A stock suspension of actomyosin was added dropwise to make a concentration of 0.12 mg/ml; this reaction mixture was gently stirred at 43°C for 5 min and then cooled to 4°C. To reverse the inactivation, dithiothreitol (DTT) (0.5 or 5.0 mM) was added at this stage. At intervals aliquots were brought to 25°C and assayed. The rate of superprecipitation was measured in the presence of 0.002 M EGTA and 1×10^{-4} M ATP with or without approximately 1×10^{-5} M free calcium (obtained by adding 0.002 M $CaCl_2$ with the EGTA).

Assay Conditions for Experiments on Clearing The experiments on clearing effects of inorganic pyrophosphate and ATP were done at higher concentrations of protein and KCl than were used in the other experiments. The conditions were: 0.1 M KCl, 0.06 M Tris, pH 7.4 at 25°C, 0.002 M EGTA, 0.001 – 0.005 M Na pyrophosphate,

5×10^{-6} M ATP, 0.5 mg/ml actomyosin, and 1×10^{-5} M free calcium when indicated.

When heat-treated protein was used in these clearing experiments, the heat inactivation was carried out with the protein and KCl at the lower concentrations given in the preceding section. After the heat treatment, the reaction mixture was allowed to stand at 4°C in a graduated cylinder until the protein settled; then the protein concentration was increased fourfold by decanting some of the supernatant, and the KCl concentration was raised to 0.1 M.

Materials ATP was obtained from the Sigma Chemical Company (St. Louis, Mo.); $AT^{32}P$, labeled equally in all three phosphates, from Schwartz Bioresearch Inc. (New York, N.Y.); dithiothreitol (DTT) from Nutritional Biochemicals Corporation (Cleveland, Ohio); creatine phosphate and creatine phosphotransferase (EC 2.7.3.2) from the Worthington Biochemical Company (Freehold, N.J.). All other chemicals were reagent grade.

RESULTS AND DISCUSSION

The Kinetics of ATP Hydrolysis and Gel Contraction From our earlier studies on the kinetics of superprecipitation, we concluded that contraction of actomyosin requires the cooperative reaction of ATP (with magnesium) at two different sites on the protein. One of these required reactions takes place at a site that catalyzes the hydrolysis of ATP, the other at a nonhydrolytic site. At the enzymatic site, the hydrolysis of ATP proceeds at the same rate whether or not the second site cooperates; but contraction results only when both reactions occur in a concerted way (7, 10).

This difference between the kinetics of hydrolysis and contraction is reflected in the top and bottom curves of Fig. 1, which show the effect of ATP concentration on the rates of these reactions at 15°C. The concentration dependence of the rate of hydrolysis (top curve) is hyperbolic; it fits the usual Michaelis-Menten mechanism in which the substrate reacts at a single kind of site, and each site operates independently. The bottom curve for contraction is sigmoid; it fits a more complex mechanism in which ATP must react cooperatively at two (or more) sites to cause contraction.

Binding of ATP to the Nonhydrolytic Site Assuming that two reactions are needed for contraction, the curves for contraction and hydrolysis in Fig. 1 can be used to derive the middle curve for the binding of ATP at the nonhydrolytic site (called site L). The derivation has been presented in detail (10). Briefly it follows this line of reasoning: each point on the simple Michaelis-Menten curve for hydrolysis indicates the fraction of protein having ATP at the hydrolytic site (this fraction is designated f_H); each point on the curve for contraction is assumed to indicate the fraction of protein having ATP at both sites—this is the product of f_H times f_L, where f_L is the fraction of protein having ATP at site L. Therefore, the values for f_L are derived

simply by dividing the values on the contraction curve ($f_H \times f_L$) by the corresponding values on the hydrolysis curve (f_H). The middle curve of Fig. 1 is a plot of f_L against ATP concentration; the curve is hyperbolic, consistent with the binding of ATP to a single site. A Lineweaver-Burk plot of these data is linear and gives the apparent dissociation constant (K_L) for ATP at site L.

Effect of Temperature on the Kinetic Parameters of Contraction The data of Fig. 1 were obtained at 15°C. Similar sets of data have been obtained at other temperatures. At each temperature, the shape of the curves is the

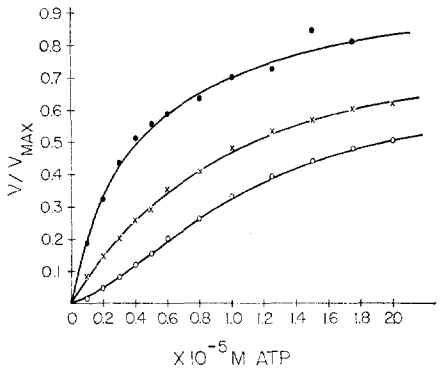

FIGURE 1. Effect of ATP concentration on the rate of ATP hydrolysis, the rate of actomyosin contraction, and the extent of ATP binding to the nonhydrolytic site (site L). Conditions are given in the text. Filled circles, rate of hydrolysis; open circles, rate of contraction; ×, extent of binding to site L. The values for the rate of contraction and the rate of hydrolysis were obtained experimentally. The values for the curve of binding to site L were derived as described in the text. 15°C.

same; i.e., hyperbolic for hydrolysis and for binding at site L, sigmoid for contraction. These data are summarized in Fig. 2.

In Fig. 2, the values for the V_{max} of hydrolysis and contraction are extrapolated from Lineweaver-Burk plots. The actual measurements were made at concentrations well below saturation to avoid the complexities of clearing and substrate inhibition (discussed in later sections). When the maximum *observable* rates are plotted instead of these extrapolated values, the Arrhenius plot is similar but steeper, especially below 15° (7), because of the increasing sensitivity to substrate inhibition as temperature is lowered.

As we have shown, and as indicated in Fig. 1, when the concentration of substrate is limiting, the rates of contraction and hydrolysis are not simply related because (we assume) two sites cooperate to cause contraction whereas one site operates independently to cause hydrolysis (7, 10). However, the parallel temperature dependence for the maximum rates of hydrolysis and

contraction, seen in Fig. 2, is consistent with the idea that the hydrolysis of ATP limits the rate of contraction when both contractile sites are saturated. This is consistent with the thesis of H. H. Weber that hydrolysis and contraction are directly related (4), and with the comparative studies of Bárány, which show that in different muscles from different animals, the rate of hydrolysis catalyzed by isolated myosin is proportional to the rate of contraction of the intact muscle (11).

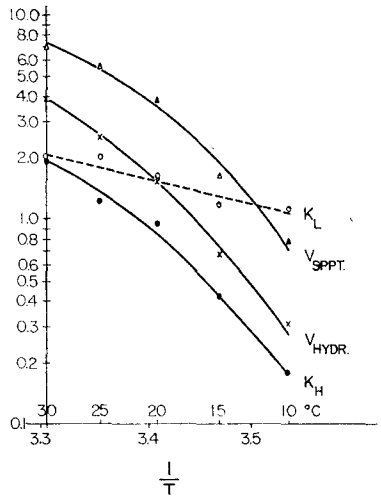

FIGURE 2. The effect of temperature on the kinetic parameters of contraction. Conditions were the standard ones described in the text. All the values were obtained from Lineweaver-Burk plots. Open triangles, maximum rate of superprecipitation; ×, maximum rate of ATP hydrolysis; filled circles, K_H, the K_M for ATP hydrolysis; open circles, K_L, the estimated dissociation constant for the binding of ATP at the nonhydrolytic site L. For hydrolysis the value on the ordinate equals micromoles P_i released/min/mg protein times 5. For K_H and K_L the value on the ordinate equals 10^5 × moles/liter. The value for superprecipitation equals OD/sec times 10.

The shallow slope of the Arrhenius plot of K_L in Fig. 2 indicates that binding of ATP to the nonhydrolytic site L involves relatively little heat change. MgATP at site L appears to us to have a permissive effect, *allowing* the protein to move, so to speak, whereas the actual driving force for the movement comes from the reaction of MgATP at the hydrolytic site.

Substrate Inhibition of Contraction and Disinhibition by Calcium In addition to the two sites at which ATP (with Mg) cooperates to cause contraction of the gel, there is a third site at which ATP (again with Mg) causes relaxation, inhibits contraction and ATP hydrolysis (12–19). A. Weber et al. (17–19) have postulated such a site to explain inhibition of contraction and hydrolysis by high concentrations of ATP and Mg; and they have shown that this in-

hibition is markedly enhanced by the removal of calcium from the protein. Our kinetic studies of gel contraction confirm this important role of calcium in controlling the extent of substrate inhibition.

When calcium binds to the protein [with a dissociation constant of about 5×10^{-6} M (17)], it profoundly modifies the inhibitory effect of MgATP. An example of this is shown in Fig. 3. At the lowest concentrations of ATP, the protein actually contracted faster without calcium; but the calcium-free protein lost this advantage at somewhat higher levels of substrate and was completely inhibited by all concentrations of ATP above 3×10^{-5} M. In contrast, the protein with calcium resisted the inhibition by substrate and contracted progressively faster with increasing concentration of ATP. Because of this great difference in sensitivity to substrate inhibition, the actomyosin gel contracts with calcium but relaxes without calcium over a wide range of ATP concentration.

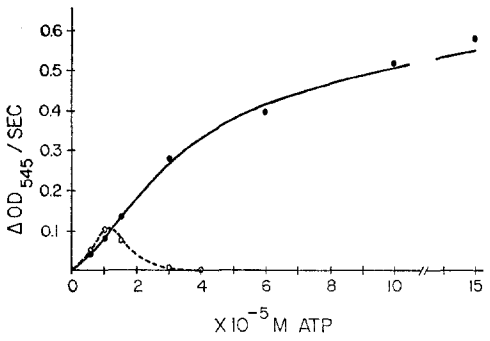

FIGURE 3. The effect of ATP concentration on the rate of contraction with and without calcium at 25°C. Conditions are those described in the text. Filled circles, with 1×10^{-5} M Ca; open circles, without Ca (0.002 M EGTA).

At each temperature (as shown in Fig. 3 for 25°C), calcium shifted the entire curve of contraction to the right, toward higher concentrations of MgATP; as a consequence, at the lowest concentrations of MgATP, the rate of contraction at each temperature was significantly faster without calcium. Apparently, the calcium-free form of the protein binds MgATP more tightly at one or both of the contractile sites; but this advantage is seen only at low levels of substrate; at higher levels of substrate it is masked by substrate inhibiton.

Substrate Inhibition in the Presence of Calcium MgATP, at high enough concentration, inhibits contraction even in the presence of an optimal level of calcium. This is most apparent at lower temperatures where the sensitivity of the protein to substrate inhibition is much greater (Fig. 4). Though the calcium form of the protein is susceptible to substrate inhibition, nevertheless, the protein at each temperature is far more easily inhibited without calcium than with it. For comparison with the rates in the presence of calcium (Fig.

4), contraction without calcium was completely inhibited by 2.5×10^{-6} M ATP at 10°C; by 8×10^{-6} M ATP at 15°C; and by 2×10^{-5} M ATP at 20°C. This effect of calcium in markedly reducing substrate inhibition is *noncompetitive*; i.e., any substrate inhibition that occurred with protein in 10^{-5} M free calcium ion (0.002 M EGTA with 0.002 M $CaCl_2$) was not relieved by increasing the concentration of free calcium ion, even tenfold.

Selective Inactivation of the Relaxing Site The most labile part of the actomyosin complex is the site at which MgATP causes relaxation. For example, as the actomyosin gel ages it loses its sensitivity to substrate inhibition, and to calcium, long before it loses its ability to contract (13, 14). The sensitivity of the gel to substrate inhibition and calcium is also lost after a number of other treatments: partial titration of SH groups (12–14, 20), mild digestion with trypsin (21), special washing procedures (22), and heat (23).

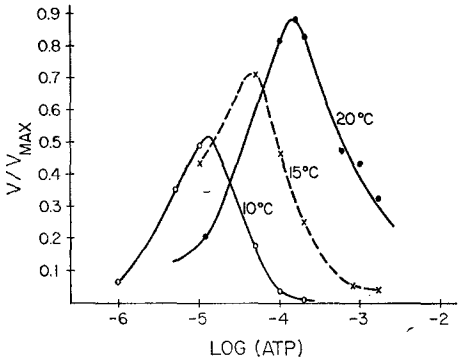

FIGURE 4. Substrate inhibition of contraction in the presence of calcium. Conditions are described in the text. The concentration of free Ca was buffered at approximately 10^{-5} M with EGTA and $CaCl_2$, both at 0.002 M. Higher concentrations of Ca at any temperature did not increase the rate of contraction at any concentration of ATP.

The trypsin-treated preparations were shown to regain their calcium sensitivity when a tropomyosin-like protein was added to them (21). This has led to the view that the function of the relaxing site probably depends on tropomyosin in association with one of the main protein constituents—most probably with the F-actin polymer (24). Certain other less well-defined proteins have also been implicated in the relaxation system (25).

We have found that heating the natural actomyosin gel for 5 min (under the conditions described in the Methods section) overcomes the calcium sensitivity in a highly selective way. As shown in Fig. 5 the heated protein was not inhibited by as much as 1.5×10^{-4} M ATP; its rate of contraction—the same with or without calcium—was about 80% of the rate of native protein with calcium. This suggests that heating does what calcium must normally do, interferes with the inhibitory action of MgATP at the relaxing site. As shown in Table I, dithiothreitol (DTT), an agent that prevents and reverses sulfhydryl oxidation (26), prevents and reverses this effect of heat.

Effect of Dithiothreitol on Heat Inactivation of the Relaxing Site Inactivation of the relaxing site was completely prevented by the presence of 0.5 mM DTT during the heat treatment. Moreover, protein that had been desensitized to substrate inhibition by heating in the absence of the reducing agent, recovered full sensitivity after 2 hr at 4°C in 5 mM DTT; or after 24 hr at 4°C in 0.5 mM DTT. The native protein was indifferent to the presence of DTT so there was no need to remove the reducing agent when testing the preparations. It has been reported that the effects of p-CMB, aging, or heat could not be reversed by certain reducing agents (13, 21). Dithiothreitol did, however, reverse the effects of aging as well as of heat.

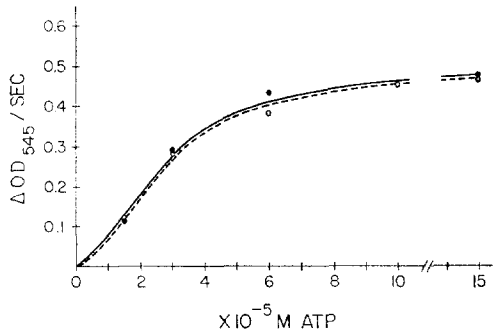

FIGURE 5. The effect of ATP concentration on the rate of contraction of heat-treated protein with and without calcium. The heat treatment for inactivating the relaxing site and general conditions for measuring the rate of contraction are described in the text. Filled circles, with 1×10^{-6} M Ca; open circles, without Ca (0.002 M EGTA).

The data indicate that heat inactivation of the relaxing site occurs because certain labile SH groups are oxidized to the disulfide form. Mueller has shown that tropomyosin has such labile SH groups and that tropomyosin sensitizes actomyosin to calcium only when these groups are in the reduced form (27). Natural actomyosin gel, extracted directly from muscle, contains tropomyosin, and in the light of Mueller's findings, it appears that the heat treatment may desensitize the gel by oxidizing the labile SH groups of tropomyosin.

Other procedures for desensitizing the protein to relaxation may break covalent bonds, remove some of the proteins, or irreversibly modify the protein. The heat treatment leaves the protein components intact and the effect is easily reversed. The ionic conditions during heat treatment are important; changes in the concentration of KCl, Mg, and Ca all affect the susceptibility of the relaxing site to the treatment. Also, higher concentrations of protein protect the relaxing site.

Clearing of the Gel by MgATP Without calcium, at the low ionic strength of these experiments (0.03 M KCl), MgATP can completely inhibit contraction without significantly changing the optical density of the gel suspension; when calcium is added to these inhibited particles they contract immediately. However, when the concentration of MgATP is raised above the level just necessary for complete inhibition or when the ionic strength (KCl concentration) is increased, another effect—called clearing—becomes apparent: the optical density of the starting gel falls and then settles at a new steady value, the higher the concentration of ATP the lower this value. Up to a point,

TABLE I
EFFECTS OF DITHIOTHREITOL ON HEAT INACTIVATION OF THE RELAXING SITE

	Rate of contraction
	OD/*sec at 545 mμ*
Heat inactivation and protection by DTT	
Unheated protein	0
Unheated protein + 0.5 mM DTT	0
Unheated protein + 0.002 M Ca	0.46
Unheated protein + 0.5 mM DTT + 0.002 M Ca	0.45
Heated protein	0.37
Heated protein + 0.002 M Ca	0.34
Protein heated with 0.5 mM DTT	0
Reversal by DTT	
Heated protein	0.39
Incubation of heated protein with 0.5 mM DTT for 24 hr	0
Incubation of heated protein with 5 mM DTT for 2 hr	0

Conditions are given in the text under the section entitled Heat inactivation of the relaxing site.

these partially cleared suspensions contract immediately when calcium is added. But when the ATP concentration is high enough, the particles completely dissociate and apparently dissolve. When calcium is added to the dissolved actomyosin, the protein eventually aggregates into visible particles and superprecipitates, but only after a relatively long time (minutes). To understand further the way in which calcium modifies the interactions between MgATP and natural actomyosin, we have studied the effects of low concentrations of calcium, e.g. 10^{-5} M, on clearing. To facilitate clearing, these experiments were done in 0.1 M KCl instead of the usual 0.03 M.

Effects of Calcium on Clearing Like ATP, high concentrations of inorganic pyrophosphate with Mg (e.g. 0.005 M MgPP) caused clearing of actomyosin

gel in 0.1 M KCl (without Mg these levels of PP had no clearing effect); and low concentrations of calcium—levels that overcome substrate inhibition—interfered with the clearing caused by MgPP. Fig. 6 shows some typical records of the fall in optical density caused by MgPP in 0.1 M KCl with and without 10^{-5} M calcium; and Table II, columns 1 and 2, gives the extent of the drop in optical density at different concentrations of MgPP, with and without this low level of calcium. The data show that the calcium form of the protein which is more resistant to substrate inhibition is also more resistant to clearing by MgPP.

ATP Potentiation of PP-Induced Clearing Without calcium, a low concentration of ATP (5×10^{-6} M) markedly potentiated the clearing effect of PP (Fig. 7). This level of ATP alone, even without calcium, caused the gel to contract slowly; yet, when it was added to 0.002 M PP, which by itself caused little or no change in optical density, the two compounds together almost completely cleared the gel. Calcium at 10^{-5} M prevented this effect of ATP; with this calcium, ATP, added to PP, caused the gel to contract rather than clear.

Effect of Heat Treatment on the Susceptibility of the Protein to Clearing Heat treatment of the protein—inactivation of the relaxing site—had much the same effect on clearing as the addition of calcium; moreover the response of the heated protein to PP and ATP was unaffected by calcium. Compared to native protein without calcium, the heated protein was less sensitive to clearing by MgPP and contracted rather than cleared when ATP was added after PP. We infer from these observations that calcium and heat interfere with clearing by interfering with the action of MgPP and MgATP at the relaxing site.

However, since contraction of the gel particles at low ionic strength can be completely inhibited by substrate without noticeable clearing of the gel suspension, it is apparent that the binding of MgATP to the relaxing site is not sufficient by itself to clear (dissociate) the particles. It is also apparent that gross dissociation of the particles is not the primary cause of substrate inhibition of gel contraction since completely inhibited particles can contract immediately in response to 10^{-5} M calcium. Instead, MgATP at the relaxing site modifies the arrangement between actin and myosin in a more subtle way; this change inhibits ATP hydrolysis and contraction. Under these conditions, when MgATP (or MgPP) binds extensively to the contractile sites, the components of the gel matrix move apart; and this separation is favored by high ionic strength. In other words, we believe that clearing is caused by the binding of MgATP to the contractile sites of the inhibited protein.

General Interpretations Certain forces, we will call them links, between the contractile elements of the isolated actomyosin gel not only hold the gel

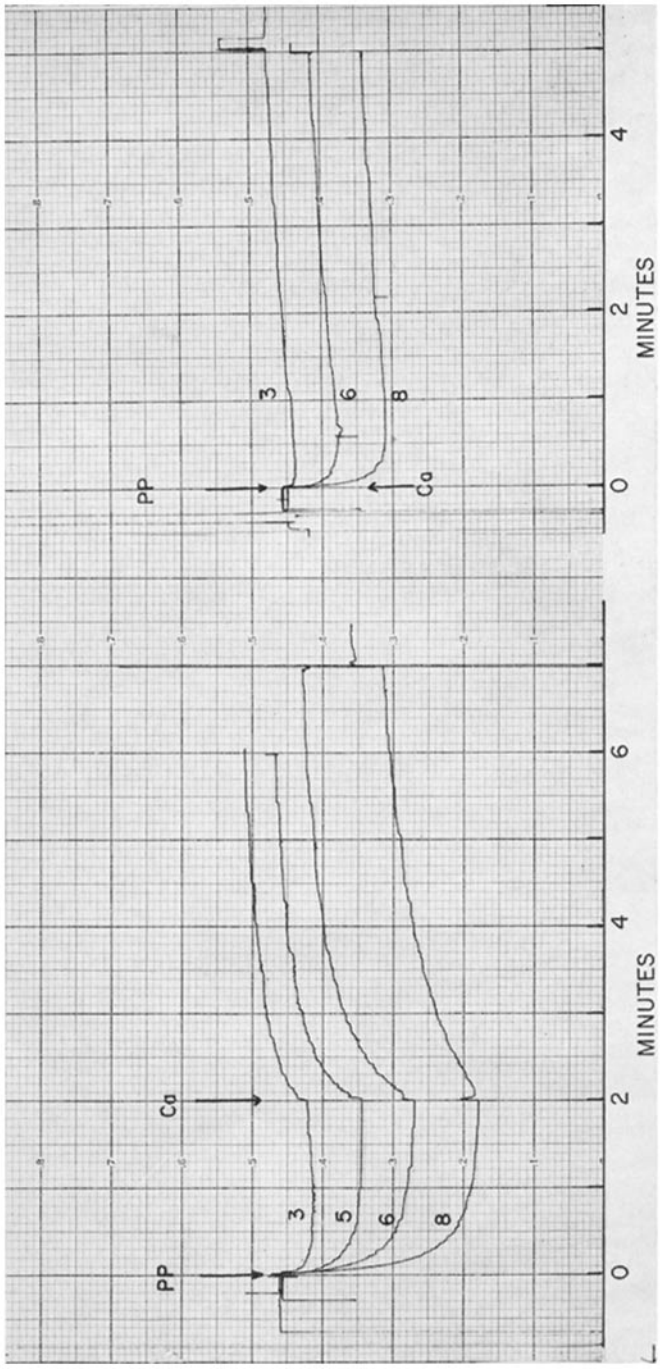

FIGURE 6. Typical records of the fall in optical density caused by pyrophosphate with and without Ca. Conditions are described in the text in the section entitled Assay conditions for experiments on clearing. The arrows indicate the time of addition of pyrophosphate or Ca. The number on each curve gives the final concentration of pyrophosphate in millimoles per liter. $CaCl_2$ was added to a total concentration of 0.002 M to give a free concentration of 10^{-5} M.

together but also prevent or retard contraction. MgATP disrupts these retarding links by binding to what we call site L; and this frees the units of the protein complex to move to a more contracted state in response to the reaction of MgATP at site H. Contraction of the gel, then, depends on two different actions of MgATP at two different sites: an action at the hydrolytic site H to provide the driving force for contraction; and an action at the nonhydrolytic site L to break links that prevent the protein from moving. Since the driving force for contraction implies the formation of new links between the contractile elements, we are led to a mechanism that describes contraction as a repeated cycle of breaking old links by the reaction of MgATP at site

TABLE II
CLEARING EFFECTS OF PYROPHOSPHATE AND ATP ON THE ACTOMYOSIN GEL

Column No.	1	2	3	4	5	6	7	8
	Extent of OD change at 545 mμ							
	Unheated protein				Heated protein			
Concentration of PP	PP alone		PP + ATP (5×10^{-6}M)		PP alone		PP + ATP (5×10^{-6}M)	
	No Ca	10^{-5}M Ca	No Ca	10^{-5}M Ca	No Ca	10^{-5}M Ca	No Ca	10^{-5}M Ca
M								
0.001	0	0	−0.26	+0.13				
0.002	−0.02	−0.01	−0.28	+0.14	−0.015	−0.015	+0.15	+0.15
0.003	−0.04	−0.02	−0.32	+0.12				
0.004	−0.065	−0.03	−0.34	+0.11	−0.055	−0.05	+0.09	+0.11
0.005	−0.11	−0.05	−0.35	+0.10	−0.08	−0.07	+0.075	+0.08

ATP was always added 3 mins after pyrophosphate. These values were obtained from records such as those shown in Fig. 7. A fall in optical density (minus sign) indicates clearing and is the maximum fall in optical density observed. A rise in optical density (plus sign) indicates contraction and the values given are those obtained 1 min after adding ATP.

L, and making new links by the reaction of MgATP at site H. (For a more detailed description of such a two-site mechanism see reference 10.)

At limiting concentrations of MgATP, the steady-state rate of contraction depends on the rate at which old links are broken as well as on the rate of ATP hydrolysis (Fig. 1). However, when the concentration of MgATP is optimal, i.e. when the rate of breaking and making links is maximal, then the rate of contraction appears to be limited by the rate of ATP hydrolysis (Fig. 2).

Contraction of the gel is controlled in part by the binding of MgATP to a relaxing site (most probably on the myosin) that is separate and distinct from the contractile sites we have just described. The binding of MgATP at this site inhibits ATP hydrolysis and gel contraction; under these conditions,

the breaking of the initial links (by the reaction of MgATP at site L) simply loosens the gel components, causing swelling and dissociation of the gel (clearing). The binding of calcium to another highly specific control site (which appears to involve tropomyosin on the actin polymer) profoundly interferes with the reaction of MgATP at the relaxing site and thereby activates contraction.

In muscle at rest the contractile filaments remain apart; they can slide freely past one another offering little resistance to stretch; and ATP is not hydrolyzed at the enzymatic sites. This resting condition depends on the removal of calcium from the sarcoplasm by the sarcoplasmic reticulum (5).

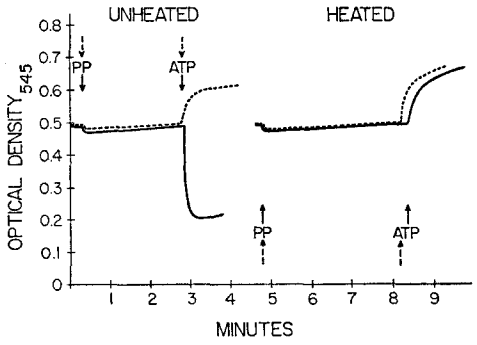

Figure 7. ATP potentiation of pyrophosphate-induced clearing. Arrows indicate time of addition of pyrophosphate or ATP to the gel. The records show the change in optical density in response to these compounds. The heated protein was treated to inactivate the relaxing site as described in the text. Pyrophosphate was added to give a final concentration of 0.002 M. ATP was added to a final concentration of 5×10^{-6} M. The extent of the optical density changes depends on the concentration of pyrophosphate; the values for other concentrations are given in Table II. Solid line, without Ca (0.002 M EGTA); dashed line, with Ca (0.002 M $CaCl_2$ + 0.002 M EGTA).

The high concentration of MgATP in this resting muscle results in binding at sites L, H, and the relaxing site, with consequent inhibition of ATP hydrolysis, and contraction, and with no linking between the actin and myosin filaments. Activation of the muscle causes contraction by making calcium available to block the action of MgATP at the relaxing site. Freed from inhibition, the filaments will then contract (slide past each other) by making and breaking force-generating links through the concerted reactions of MgATP at sites H and L.

Note Added in Proof Whereas calcium (at micromolar concentrations) antagonizes substrate inhibition of contraction, we have recently been able to show that free magnesium (at millimolar concentrations) potentiates the inhibitory effect of MgATP. In these opposite actions, calcium and magnesium are *noncompetitive*; the results

suggest still another effector site in the control system to which magnesium binds. These findings will be presented in a later paper.

Preliminary reports of some of this work have been presented (references 1 and 2).
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