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Regulation of Muscle Contraction. Effect of Calcium on the Affinity of Troponin for Actin and Tropomyosin†

Sarah E. Hitchcock

ABSTRACT: The calcium dependence of the reconstituted actomyosin adenosine triphosphatase (ATPase) depends on the troponin concentration. Reconstituted actomyosin was made from rabbit actin and myosin and increasing amounts of chicken tropomyosin and troponin. The ATPase was measured at different calcium ion concentrations from 1.4×10^{-9} to 1.4×10^{-6} M in the presence of Mg^{2+} (pH 7.5). When the data are normalized, it is seen that the calcium concentration required for half-maximal activity increases from 3.6×10^{-8} to 1.7×10^{-7} M with a ninefold increase in troponin concentration. Half-maximal calcium binding by troponin is at 6.5×10^{-8} M Ca^{2+} in the presence of 2 mM $MgCl_2$ at pH 7.5. An explanation for the observed shift in calcium dependence is that troponin in the absence of calcium has a higher affinity for actin–tropomyosin than troponin with calcium. This interpretation is supported by a mathematical analysis of this multiple equilibria system in the Appendix accompanying the paper. Measurement of exchange between free and actin-bound troponin is consistent with the assumption that the system is at equilibrium.

Troponin, a regulatory protein in the myofibril, binds calcium and confers a calcium-sensitive inhibition on actomyosin ATPase and superprecipitation. This inhibition is considered the *in vitro* analog of relaxation in the myofibril (Ebashi and Kodama, 1965). Troponin is associated with actin and tropomyosin in the thin filaments of vertebrate striated msucle (Ohtsuki *et al.*, 1967). Calcium stimulates contraction by combining with troponin without any known direct effect on myosin. An important question is how troponin transmits information about the presence or absence of calcium to actin and myosin resulting in contraction (ATPase) or relaxation (inhibition of ATPase), respectively. Kinetic experiments have shown that troponin reduces the affinity of actin for myosin in the presence of EGTA[1] (Parker *et al.*, 1970; Eisenberg and Kielley, 1970) and that troponin's effect is mediated through actin and tropomyosin rather than myosin (Weber and Bremel, 1971; Spudich and Watt, 1971). Structural studies of native and synthetic thin filaments and X-ray diffraction of muscle have shown that in the presence of calcium, tropomyosin lies in the groove of the F-actin helix (Moore *et al.*, 1970; Spudich *et al.*, 1972) and in relaxing conditions it shifts out of the groove possibly to the position of myosin attachment on actin (Hanson *et al.*, 1973; Huxley, 1973; Parry and Squire, 1973).

The present study is concerned with the interaction of troponin with actin, tropomyosin, and myosin with particular attention to the effect of calcium. Evidence is presented which suggests that calcium reduces the affinity of troponin for the actin–tropomyosin complex. It is possible that this change in affinity is a fundamental property of the calcium-sensitive regulation of contraction in the myofibril. The tight binding of troponin to actin and tropomyosin in the absence of calcium may be directly involved in keeping tropomyosin out of the groove of the actin helix, a position found only in the specific conditions of relaxation.

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[1] Abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl-ether)-*N,N'*-tetraacetic acid.

Experimental Section

Preparation of Proteins. Troponin and tropomyosin were made from breast muscle of White Rock chickens weighing 5–6 lb obtained at Mayflower Poultry, Cambridge, Mass.

Troponin was prepared according to Ebashi and Ebashi (1964) with minor modifications. The water extraction of relaxing factor following extraction of myosin from the minced muscle was performed overnight in the cold in the presence of 1 mM dithiothreitol (Calbiochem). The extract was collected by centrifugation for 10 min at 10,000*g* (Sorvall RC2) and the supernatant was clarified by centrifugation for 1 hr at 60,000*g* (Spinco Model L ultracentrifuge). Ammonium sulfate fractionation (Mann, enzyme grade) was performed in the cold and the 40–60% precipitate was collected by centrifugation and dialyzed *vs.* 0.05 M NaCl–0.01 M imidazole (pH 7.0)–0.5 mM dithiothreitol and clarified by centrifugation for 1.5 hr at 60,000*g*. This fraction was called relaxing protein and contains mostly tropomyosin and troponin. Troponin was purified from relaxing protein by isoelectric precipitation of tropomyosin. The protein concentration of the relaxing factor was adjusted to about 2–3 mg/ml and the NaCl concentration brought to 1 M by addition of 4 M NaCl. The pH was lowered to pH 4.6 with 7.5% acetic acid and the precipitate containing tropomyosin was removed by centrifugation for 10 min at 35,000*g* in a Sorvall RC2 centrifuge. The supernatant was neutralized with 2 M NaOH and dialyzed *vs.* 0.05 M NaCl–0.01 M imidazole–0.5 mM dithiothreitol (pH 7.0). The troponin was concentrated to 4–5 mg/ml by ultrafiltration (Amicon, UM-20E or PM-10 membrane) and clarified by centrifugation for 1 hr at 60,000*g*. From 200 g of muscle one recovers about 50 mg of troponin. Both the relaxing protein and troponin were stored frozen (−20°) for periods of 1–2 months. Chicken troponin has three components: 44,000, 23,000, and 19,000 daltons, as determined by sodium dodecyl sulfate acrylamide gel electrophoresis, similar to, but not identical with, those found in rabbit (Greaser and Gergely, 1971).

Chicken tropomyosin was prepared according to Bailey (1948) as modified by Lehman and Szent-Györgyi (1972). It gives a single band on sodium dodecyl sulfate gel electrophoresis without significant contamination by troponin. It was stored frozen at −20°.

Rabbit actin acetone powder was prepared according to Straub (1942) and purified using the procedure of Mommaerts (1952). It was stored as an acetone powder at −20°. The acetone powder was extracted at 0° with CO_2 free water and the actin was polymerized once at room temperature with 10 mM NaCl and 0.5 mM $MgCl_2$ to reduce tropomyosin–troponin impurities (Drabikowski and Gergely, 1964). The actin in the pellets (100,000*g*, 3 hr) gives a single band on sodium dodecyl sulfate gels. Rabbit myosin was prepared according to Szent-Györgyi (1951) as modified by Mommaerts and Parrish (1951) and stored in 50% glycerol at −20°. Before use, an aliquot of myosin was dialyzed *vs.* 0.05 M NaCl–5 mM phosphate buffer (pH 6.2). The precipitate was collected, dissolved in 0.6 M NaCl–5 mM phosphate buffer (pH 7.0) and clarified.

ATPase Measurements. ATPase activity was measured at 25°, pH 7.5, in a pH-Stat with an automatic titrator (Titrigraph Type SBR 2C, titrator-type TTT 1C, Autoburette ABU 11, Radiometer, Copenhagen). NaOH (0.04 M) was used as a titrant and it was assumed that 1 μmol of NaOH added represents 1 μmol of ATP hydrolyzed to ADP + P_i (Green and Mommaerts, 1953). For the Mg-activated ATPase of actomyosin, the final assay mixture was 10 ml of 2 mM $MgCl_2$–40 mM NaCl–0.7 mM ATP. Appropriate mixtures were made of CaEGTA and EGTA, to a final concentration of 0.2 mM, to obtain free calcium concentrations between 10^{-5} and 10^{-8} M assuming the dissociation constant for CaEGTA at pH 7.0 is 1.9×10^{-7} M (Chaberek and Martell, 1959). When corrections are made for the pH, Mg^{2+}, and salt conditions used in the present ATPase experiments, the dissociation constant is 2.7×10^{-8} M (Regenstein, 1972). This dissociation constant has been used to calculate free calcium concentration.

The proteins used in the actomyosin assay were combined as follows: actin (in 0.05 M NaCl), tropomyosin (in 0.05 M NaCl), troponin (in 0.05 M NaCl–0.01 M imidazole–0.5 mM dithiothreitol, pH 7.0). The proteins were added in the following ratio (weight/weight): 5 myosin:1 actin:0.33 tropomyosin (when constant), troponin varied. At a 5:1 ratio the myosin is in excess over actin and therefore all the actin is activating the myosin ATPase. This actin–tropomyosin–troponin mixture was brought to a volume of 0.5 ml, made 0.6 M in NaCl by addition of 4 M NaCl, and myosin (0.1 ml in 0.6 M NaCl) was added and mixed on a Vortex-Genie. The actomyosin was added dropwise to the assay mixture and the reaction was initiated by addition of ATP. Rate measurements were corrected for drift when present.

Calcium Binding Measurements. Calcium binding by troponin was measured by equilibrium dialysis. Protein was dialyzed to equilibrium *vs.* 0.05 M NaCl, 0.01 M imidazole, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (pH 7.5), and 25 μM ^{45}CaEGTA with free calcium concentrations varying from 10^{-9} to 10^{-6} M assuming the dissociation constant at pH 7.5 is 2.7×10^{-8} M. Two 0.1-ml samples were each combined with 6 ml of scintillation fluid containing Beckman Bio-Solv–toluene (1:5) containing 0.5% 2,5-diphenyloxazole and 0.03% *p*-bis[2-(phenyloxazoyl)]benzene (Packard) and counted in a Beckman LS-255 liquid scintillation counter. A third 0.1-ml aliquot was used for protein determination.

Exchange Experiments. Troponin (3 mg/ml) was labeled with 1 mM *N*-ethyl-[1-^{14}C]maleimide (5 Ci/mol) in 0.1 M phosphate buffer (pH 7.0) for 10 min at room temperature according to Riordan and Vallee (1972). The protein was applied to a Sephadex G-25 column equilibrated with 50 mM NaCl, 10 mM imidazole, 2 mM $MgCl_2$, 0.2 mM CaEGTA + EGTA, free calcium concentration 1.4×10^{-7} M, and 0.5 mM dithiothreitol (pH 7.5) to remove unbound label. The protein was dialyzed against several changes of buffer. The radioactivity in the dialysates was at the background level showing that the label is tightly bound to the troponin. The ability of labeled troponin to confer calcium sensitivity to the actomyosin ATPase is indistinguishable from that of untreated troponin.

Radioactive troponin (4 mg) was combined with F-actin (10 mg) and tropomyosin (3.3 mg), diluted with buffer to a final actin concentration of 0.5 mg/ml, and sedimented for 3 hr at 100,000*g*. The pellet was resuspended and sedimented a second time. The first and second supernatants contained 7 and 2% of the initial radioactivity. The final complex had 33,370 cpm/mg of protein.

The actin–tropomyosin–troponin complex was combined with cold troponin, and myosin in 0.2 ml as described for the ATPase measurements, diluted with 1.8 ml of the above buffer without NaCl to precipitate the actomyosin, and sedimented for 20 min at 40,000*g* in a Sorvall RC2 centrifuge. The radioactivity of two 0.5-ml aliquots of the supernatant was measured as described for the calcium binding measurements.

Protein Determination. Protein was determined by the pro-

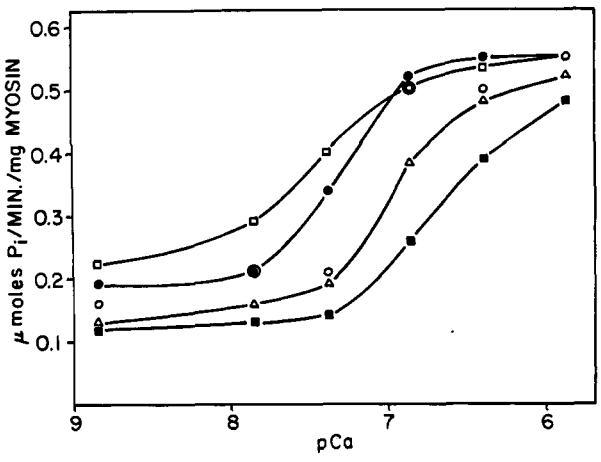

FIGURE 1: The calcium dependence of the actomyosin ATPase at different troponin concentrations. The assay conditions are described in Experimental Section. Each assay contains 0.85 mg of myosin, 0.17 mg of actin, and the amount of troponin:tropomyosin was varied in a 1.5:1 ratio in 10 ml. Symbols are (□) 0.10 mg of troponin; (●) 0.15 mg; (○) 0.20 mg; (▲), 0.45 mg; (■) 0.90 mg. The same shift in calcium dependence is observed when A:TM is constant at 3:1 (w/w) and troponin alone varied. The ATPase of actomyosin (±tropomyosin) at 10^{-9} and 10^{-6} M Ca^{2+} is 0.34 μmol of P_i/min per mg of myosin.

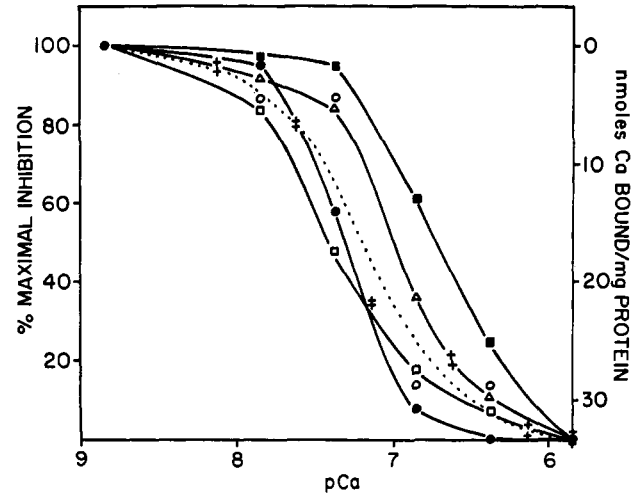

FIGURE 2: The calcium dependence of the actomyosin ATPase at different troponin concentrations: normalized data. The data from Figure 1 were normalized by taking the ATPase activity at 1.4×10^{-6} M Ca^{2+} as 0% maximal inhibition and that at 1.4×10^{-9} M Ca^{2+} as 100% maximal inhibition. The symbols are the same as for Figure 1. The dotted line (+) is calcium binding by troponin (a different preparation than that used for the ATPase measurements) in the absence of actin + tropomyosin.

cedure of Lowry *et al.* (1951) using bovine serum albumin, calibrated by the microkjeldahl technique, as a standard.

Chemicals. All chemicals were of reagent grade and were purchased from special suppliers where indicated. $^{45}CaCl_2$ and *N*-ethyl-[1-^{14}C]maleimide were purchased from New England Nuclear. Glass-distilled water was used in making all solutions.

Results

The calcium dependence of the reconstituted actomyosin ATPase has been measured at different troponin concentrations. Results of a representative experiment in Figure 1 show the unexpected result that the per cent inhibition at a given calcium concentration depends on the troponin concentration. The higher the troponin concentration, the higher the free calcium concentration required to relieve inhibition. The simplest result would be if the calcium dependence of the ATPase were independent of troponin concentration assuming the actin regulated by troponin is either inhibited or not, depending only on the calcium concentration. The shift in calcium dependence suggests that calcium is having a more complex effect on actin–tropomyosin–troponin interactions. With higher troponin concentrations at 1.4×10^{-6} M Ca^{2+} a small amount of inhibition remains relative to the ATPase at lower troponin concentrations. It is not relieved by addition of more calcium. At all troponin concentrations the ATPase at 1.4×10^{-6} M Ca^{2+} is activated ~60% relative to that of actin, myosin, and tropomyosin alone. This activation has been reported by other laboratories (*cf.* Greaser and Gergely, 1971).

The data from Figure 1 have been normalized by taking the ATPase activity at 1.4×10^{-6} M Ca^{2+} as 0% inhibition and that at 1.4×10^{-9} M Ca^{2+} as 100% inhibition and are replotted as per cent maximal inhibition in Figure 2. It is seen more clearly that the calcium concentration required for half-maximal activity (inhibition) increases with the troponin concentration. At low troponin concentrations the calcium concentration required for half-maximal inhibition is ~4.2×10^{-8} M (pH 7.5), slightly lower than that for half-maximal calcium binding at pH 7.5 (6.5×10^{-8} M, Figure 2). As the troponin concentration is increased, the calcium concentration at which inhibition is half-maximal increases about fivefold with a ninefold increase in troponin concentration (in a range approaching maximal inhibition of the actomyosin ATPase at 1.4×10^{-9} M Ca^{2+}). As noted above, the ATPase activity at 1.4×10^{-6} M Ca^{2+} is greater in the presence than in the absence of troponin. The activated values have been used for 0% inhibition in the normalization because they are relatively constant over the range of troponin concentration of interest. The relationship of activation to calcium sensitivity, if any, is unclear at this time.

At pH 7.5 in conditions similar to those used in the ATPase measurements, half-maximal calcium binding by troponin is at 6.5×10^{-8} M Ca^{2+}, slightly higher than that required for half-maximal inhibition at the lower troponin concentrations (Figure 2). It is not known if this difference is significant.

An important assumption made in interpreting these data is that the actin–tropomyosin–troponin interactions are at equilibrium. The exchange between troponin bound to actin–tropomyosin with free troponin has been measured in the following manner. Troponin, labeled with *N*-ethyl-[1-^{14}C]-maleimide, was combined with actin and tropomyosin. This radioactive complex was combined with cold troponin and myosin, precipitated, and sedimented as described in the Experimental Section. Radioactivity in the supernatant was measured (Figure 3). The per cent total radioactivity in the supernatant increases with free troponin concentration from 38% without additional troponin to a maximum of 84%. The exchange of radioactivity occurs in the presence and absence of ATP, is independent of the calcium ion concentration, and the reaction is completed within the minimum time required to perform the experiment (<2 min before centrifugation). The radioactivity is also exchanged in the absence of myosin. The radioactivity in the supernatant in the absence of cold troponin is probably due to solubilization of the actomyosin-relaxing protein complex at the low protein concentrations used (similar to the ATPase conditions) since it is dependent

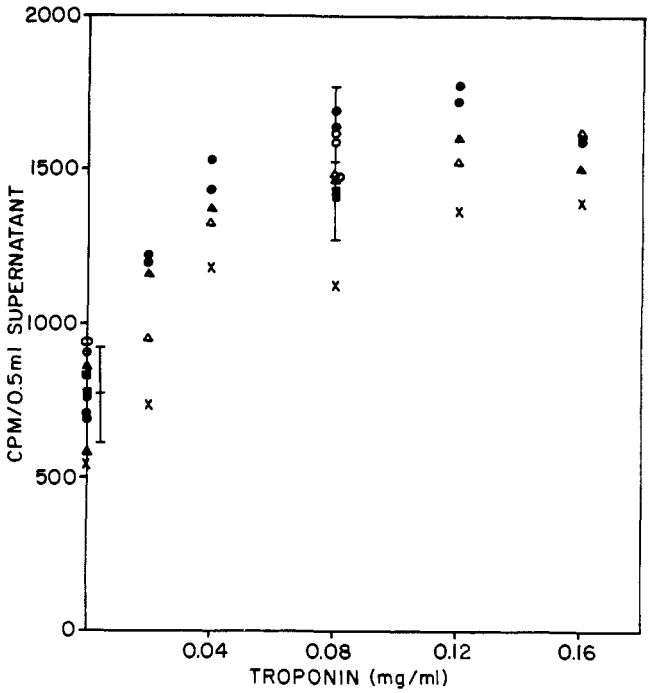

FIGURE 3: Exchange of radioactivity as a function of troponin concentration. The experiments were performed as described in the Experimental Section. Each point represents one measurement; each type of symbol a different experiment: (●, ▲, ■) actin–tropomyosin–troponin–myosin, no ATP, 1.4×10^{-7} M Ca^{2+}; (△) same as filled symbols in the presence of 1 mM ATP; (○) same as filled symbols but $[Ca^{2+}]$ varied, 1.4×10^{-9}, 1.4×10^{-8}, 1.4×10^{-6} M; (×) actin–tropomyosin, troponin, no myosin, no ATP, 1.4×10^{-7} M Ca^{2+}. The bars indicate $\bar{x} \pm s$ for the ●, ▲, ■ measurements.

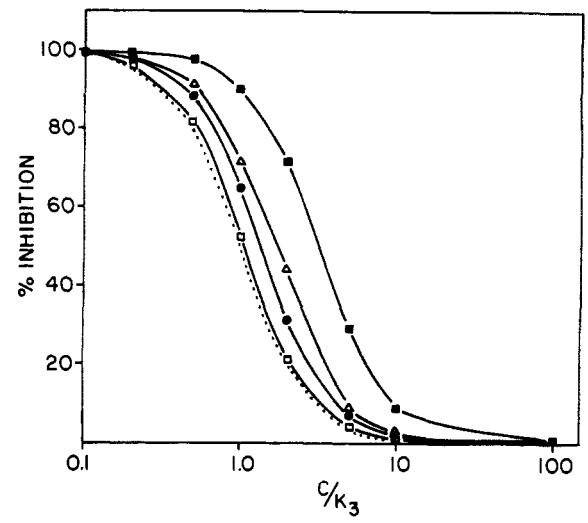

FIGURE 4: Per cent inhibition *vs.* $(Ca)/K_3$, normalized curves at $n = 2$. Dotted line: $K_1/K_2 = 1$. All values of $K_1/(T_f)$ normalize to here. Solid lines: $K_1/K_2 = 0.1$ at different values of $K_1/(T_f)$: ■, $K_1/(T_f) = 0$ (saturation); △, $K_1/(T)_f = 0.5$; ●, $K_1/(T_f) = 1$; □, $K_1/(T_f) = 10$.

on protein concentration and the label is not removed from troponin by extensive dialysis.

One possible trivial explanation has been satisfactorily eliminated. The shift seen in Figure 2 would be expected if Ca-free troponin were added and the calcium in the buffer depleted. This is unlikely since the maximum amount of calcium bound by troponin is 25 nmol whereas there are 2 μmol of CaEGTA + EGTA buffer (0.10–1.97 μmol of calcium). Also, when the troponin is preequilibrated with the CaEGTA buffer by dialysis, the same calcium shift occurs. In order to compare the experimental results with the theoretical curves discussed in the Appendix, it is necessary to know the relationship between stoichiometric binding of troponin to actomyosin and the experimental and theoretical saturation of the calcium sensitivity of the ATPase. An attempt has been made to answer this question by sedimenting actomyosin with increasing amounts of troponin in conditions similar to those in the ATPase experiments. Sodium dodecyl sulfate acrylamide gels of the pellets are of too poor resolution to accurately measure the additional troponin bound.

Discussion

An explanation for the shift in calcium dependence of the actomyosin ATPase with increased troponin concentration is that troponin in the absence of calcium has a higher affinity for actin (plus tropomyosin) than troponin saturated with calcium. If the affinities were the same, there would be no shift.

It is interesting to speculate about what effect calcium has on troponin which results in reduced affinity of troponin to actin–tropomyosin and to consider the consequences on the mechanism of troponin action. Calcium may regulate contraction by altering the binding of troponin to actin–tropomyosin thereby influencing the actin–myosin interaction by direct steric blocking of the active sites or conformational changes in the thin filament. A two-site model can explain the affinity change. This model proposes that troponin has two different binding sites, one which is calcium sensitive and is formed in the absence of calcium and broken in the presence of calcium and a second binding site which is not calcium sensitive and is formed all the time. The reduction in the affinity constant (as measured by ATPase activity) caused by calcium does not imply that troponin dissociates from the actin filament. Structural requirements for regulation make it unlikely that the troponin is free in the cytoplasm during contraction.

The calcium-dependent binding of troponin components to actin–tropomyosin has been demonstrated directly using the preparative ultracentrifuge (Hitchcock *et al.*, 1973). Troponin-I + C (Greaser *et al.*, 1973) bind to actin–tropomyosin in 0.1 mM EGTA but dissociate in 0.1 mM CaEGTA (in the presence of 2 mM $MgCl_2$). The addition of troponin-T to the troponin-I + C restores the binding in the presence of calcium suggesting that troponin-T attaches troponin to the thin filament in the presence as well as in the absence of calcium forming the calcium-insensitive binding site. The calcium-sensitive site is in the troponin-I + C attachment to actin–tropomyosin.

In interpreting the ATPase experiments we have made the following assumptions. (The abbreviations are those used in the Appendix.) (1) That there are two functional forms of troponin (troponin–tropomyosin): T which is not fully saturated with calcium and when bound to actin is capable of inhibiting the Mg^{2+}-activated actomyosin ATPase and TCa_n which is fully saturated with calcium and when bound to actin does not inhibit the Mg^{2+}-activated actomyosin ATPase. (2) That the per cent inhibition of the Mg^{2+}-ATPase before normalization is proportional to the percentage of total actomyosin (A_0) in the form of TA (actin with T on it). $((TA)/(A_0) \times 100 =$ % inhibition.) This is before normalization. An implication of this assumption is that A is functionally, and therefore considered mathematically equivalent to TCa_nA. (3) That the system is at equilibrium. The results of the experi-

ments to measure exchange between actin-bound and free troponin are consistent with this assumption.

The observed shift in the calcium dependence of the actomyosin ATPase would be expected if the T form of troponin has a higher affinity for actin than TCa_n and is therefore preferentially bound. At a given calcium concentration, a certain fraction of the troponin is in the T form, and the higher the troponin concentration a greater proportion of the actin has T rather than TCa_n on it. Since the percentage inhibition has been assumed to depend on the percentage of TA, the inhibition at a given calcium concentration will depend on the troponin concentration.

The conclusion that calcium reduces the affinity of troponin for actin is consistent with the mathematical analysis of this multiple equilibria system in the Appendix accompanying this paper. (The four equilibria are described in eq 1–4 in the Appendix. For notation, see the Appendix.[2]) The mathematical analysis shows that the calcium dependence of the actomyosin ATPase (slope and position of the % inhibition *vs.* pCa $[(Ca)/K_3]$ curves) shows no change with increasing troponin concentration when the affinity of troponin for actin is the same in the presence and absence of calcium ($K_1 = K_2$). When T has a higher affinity than TCa_n for A ($K_1 < K_2$), the calcium ion concentration required for a given percent inhibition increases with increasing troponin concentration like the shift in calcium dependence of ATPase activity observed experimentally (Figure 2).

Theoretical curves, at different values of n and K_1/K_2, can be compared with the experimental data. The value of n, the number of moles of calcium bound per mole of troponin during the transition from the inhibited to the uninhibited form, is fewer than the total moles bound (4 to 5, Bremel and Weber, 1972) and is a measure of the cooperativity of the system. The theoretical curves when $K_1/K_2 = 0.1$ and $n = 2$ (Appendix, Figure 4) compare qualitatively with the experimental curves (Figure 2). It is also possible to do more complete calculations using a curve-fitting program from the equations in the Appendix. However, it is not appropriate to estimate values for K_1/K_2 and n since the data are qualitative and there are difficulties in relating experimental and theoretical parameters. For example, 100% inhibition (unnormalized value) is never reached experimentally. For this reason there is a problem in relating experimental troponin concentrations to $K_1/(T_f)$ or $K_1/(T_0)$, a measure of saturation. Also, the activation of the actomyosin Mg^{2+}-ATPase by troponin has not been explicitly considered. The important conclusion is that the interpretation of the kinetic data is consistent with the mathematical analysis of this multiple equilibria system.

The reduced affinity of troponin for actin in the presence of calcium can be related mathematically to the two-site model if it is assumed that K_1 has calcium-sensitive (K_{Ca}) and calcium-insensitive components while K_2 only has the calcium-insensitive component ($K_1 = K_{Ca}K_2$). Since troponin is a multicomponent system, one may expect more than one binding site to the thin filament complex. Troponin can interact with tropomyosin (Nonomura *et al.*, 1968; Sugita *et al.*, 1969; Cohen *et al.*, 1972) and actin alone (Parker *et al.*, 1970; Schaub and Perry, 1971). It is not yet known if the calcium-sensitive binding of troponin is to actin or tropomyosin.

One prediction of the mathematical analysis is that if $K_1 < K_2$, then the calcium dependence of the ATPase of actomyosin nearly saturated with troponin (the higher troponin concentrations used in the ATPase experiments) would differ from the calcium dependence of the calcium binding by free troponin. When calcium binding by free troponin is measured at pH 7.5, the calcium ion concentration required for 50% maximal binding by troponin (6.5×10^{-8} M) is similar to that required for 50% maximal ATPase activity at the lower troponin concentrations used ($\sim 4.2 \times 10^{-8}$ M, Figure 2). It is not known if these values are significantly different, but they are clearly lower than the calcium concentration required for 50% maximal activity with the highest troponin concentration (1.7×10^{-7} M). Weber and Bremel (1971) showed that the calcium ion concentration required for 50% maximal calcium binding by myofibrils is less than that needed for 50% maximal ATPase activity (at pH 7.0).

Acknowledgments

I am indebted to Dr. Andrew G. Szent-Györgyi, in whose laboratory this work was performed, for originally suggesting the ATPase experiment. I am grateful to him, Dr. Joe M. Regenstein and Eva M. Szentkiralyi, for many spirited and stimulating discussions during the course of the work and preparation of the manuscript. I thank Dr. Hugh E. Huxley for his helpful criticisms of the manuscript.

Appendix: Mathematical Analysis of a Multiple Equilibria System: Troponin–Tropomyosin–Actomyosin Interactions with and without Calcium[3]

By Joe M. Regenstein[4,*] and Sarah E. Hitchcock[5]

This appendix provides the mathematical justification for the statement in the accompanying paper that the observed data are consistent with the interpretation that actomyosin has a stronger affinity for relaxing protein in the inhibiting form than in the noninhibiting calcium saturated form.

The multiple equilibria system analyzed consists of the following components: an enzyme (myosin ATPase), activator (actin), and a ligand binding inhibitor (relaxing protein, troponin–tropomyosin, which binds calcium) which affects the activator but does not act directly on the enzyme. The two equilibria being compared are the activator–inhibitor interaction and the interaction of the ligand with the inhibitor. The values of each of the individual equilibrium binding constants of the system can be determined by measuring the activator–enzyme interaction (actin–activated myosin ATPase) as a function of free ligand concentration.

[2] In the notation used in the Appendix, A denotes the actomyosin complex and T the troponin–tropomyosin. The abbreviated nomenclature is used in the Appendix for simplicity. In this paper troponin action is discussed in terms of interactions with actin and tropomyosin rather than myosin, but we do not know if the equilibrium affected by calcium is the binding of tropomyosin–troponin to actin or that of troponin to actin–tropomyosin.

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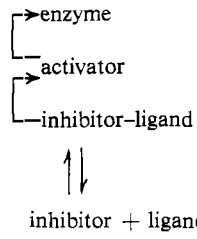
The system can be diagrammed as follows



The data in the accompanying paper were obtained by measuring the ATPase activity of the reconstituted actomyosin at different calcium ion concentrations and it is assumed that the amount of inhibition of the Mg-activated actomyosin ATPase observed is a direct measure of the inhibited complex. It is further assumed that the interactions involve only two forms of relaxing protein, the inhibitory form which is not fully saturated with calcium and the noninhibitory form which is saturated with calcium. If the number of moles of Ca bound per mole of relaxing protein is greater than one, it is assumed that the transition between the two forms can be described as a single reaction with a dissociation constant (K_3).

Given these conditions, the following six equations interrelate the different species in equilibrium when the calcium ion concentration and the concentration of all protein species remains the same but ATP is hydrolyzed at a constant rate.

$$TA \rightleftharpoons T + A \qquad K_1 = (T)(A)/(TA) \tag{1}$$

$$TCa_nA \rightleftharpoons TCa_n + A \qquad K_2 = (TCa_n)(A)/(TCa_nA) \tag{2}$$

$$TCa_n \rightleftharpoons T + nCa \qquad K_3 = (T)(Ca^n)/(TCa_n) \tag{3}$$

$$TCa_nA \rightleftharpoons TA + nCa \qquad K_4 = (TA)(Ca^n)/(TCa_nA) \tag{4}$$

$$T_f = T + TCa_n[6] \tag{5}$$

$$A_0 = A + TA + TCa_nA \tag{6}$$

where K_i = dissociation constant, T = relaxing protein (troponin–tropomyosin complex), A = actomyosin complex, Ca = calcium, n = number of moles of calcium bound during the transition from the inhibited to the noninhibited form, A_0 = total concentration of actin, and T_f = free troponin.

In this formulation it is assumed that only relaxing protein (T) that interacts with the actomyosin (A) can affect the ATPase and that the inhibition is directly proportional to the amount of AT formed (with the A or $ATCa_n$ forms being equally active). The percentage inhibition is measured by the following equation

$$\% \text{ inhibition} = (TA)/(A_0) \times 100 = [(A_0)/(TA)]^{-1} \times 100 \tag{7}$$

The latter form will be simpler mathematically.

The value of the % inhibition can be calculated from the dissociation constants and the concentration of components added: Ca, T_f, A_0 (please see footnote 6). Starting with eq 7 and substituting the value of A_0 from eq 6 gives

$$(TA)/(A_0) = (TA)/[(A) + (TA) + (TCa_nA)] \tag{8}$$

Equation 8 can be solved by obtaining values from eq 1 to 5 for TA and TCa_nA

$$(TA) = \frac{(A)K_3(T_f)}{K_1[(Ca^n) + K_3]} \tag{9}$$

$$(TCa_nA) = \frac{(A)Ca^n(T_f)}{K_2[K_3 + (Ca^n)]} \tag{10}$$

These values are then substituted into equation 8 to give:

$$\frac{(TA)}{(A_0)} = \frac{1}{\frac{(Ca^n)}{K_3}\left(\frac{K_1}{(T_f)} + \frac{K_1}{K_2}\right) + \frac{K_1}{(T_f)} + 1} \tag{11}$$

Equation 11 makes certain predictions. When $n = 1$, $(Ca)/K_3$ equals 1 when the calcium concentration is equal to the binding constant of the free relaxing protein. K_3 is directly measurable by calcium binding. The term K_1/K_2 is the ratio of the dissociation constants of relaxing protein without and with calcium from the actomyosin complex. If these are equal, $K_1/K_2 = 1$. Our postulate is that they are not equal and specifically that $K_1 < K_2$. The term $K_1/(T_f)$ is a measure of saturation of the actomyosin by the inhibited relaxing protein. Before saturation is reached, the value of this term is positive and at saturation it becomes zero.

When the % inhibition is calculated as a function of $(Ca)/K_3$ at different values of $K_1/(T_f)$ when $K_1 = K_2$, the only point where a 50% inhibition of Mg-ATPase occurs at $Ca = K_3$ is when the troponin is fully saturating ($K_1/(T_f) = 0$) and 100% inhibition occurs when no calcium is present. With less than saturating troponin ($K_1/(T_f) > 0$), one never reaches full inhibition at any measurable calcium concentration.

It is possible to normalize the data for the various levels of saturation by calculating the maximum inhibition obtained (at Ca = 0) and then dividing the observed values by this value $([(TA)/(A_0)]/[(TA)/(A_0)]_{Ca/K_3=0})$. Normalizing the data by this procedure leads to an important result. If $K_1 = K_2$, at all values of $K_1/(T_f)$ the normalized curves superimpose (Figure 4). Using eq 11 and normalization, it can be shown that the value of the normalized inhibition is independent of T_f and equals $[(Ca^n)/K_3] + 1$.

However, if K_1/K_2 does not equal 1, then one obtains a family of normalized curves at different $K_1/(T_f)$ as shown in Figure 4. At saturation ($K_1/(T_f) = 0$), the value of 50% inhibition shifts in proportion to the value of K_1/K_2 (K_1/K_2 =

[6] We have simplified the mathematical analysis by solving for free troponin instead of total troponin. The result is that Figure 4 is a function of free troponin whereas Figure 2 of the main paper is of total troponin (T_0). The following equations relate the two quantities:

$$T_0 = T_f + TA + TCa_nA$$
$$= T_f + A_0 - A$$

A_0 is known but A is not. However, in the region of interest, T_f is greater than A_0 and the value of A is small, probably less than 30% of A_0 at the lowest troponin concentrations and approaching 0% of A_0 at the higher concentrations. This simplification does not affect the basic conclusions we have made regarding the effect of calcium on the affinity of troponin for actomyosin.

A computer program gives explicit solutions to eq 1–6. $(TA)/(A_0)$ as a function of $(Ca)/K_3$ can be calculated for different values of $(T_0)/(A_0)$, $(A_0)/K_1$, K_1/K_2, and n. The curves obtained from the program are in qualitative agreement with both the experimental data and the analysis of this appendix.

1.0, 0.1 shown). At 50% inhibition ($A_0/(TA) = 2$) and saturation ($K_1/(T_f) = 0$) eq 11 becomes

$$(Ca_{50\%})^n = K_3\frac{K_2}{K_1} \tag{12}$$

where $Ca_{50\%}$ = the calcium concentration at which 50% inhibition is observed under the conditions specified.

The result for $K_1 < K_2$ in Figure 4 is as expected: if the inhibiting form of troponin is more strongly bound, the inhibition at saturation is not relieved until a higher calcium concentration is reached. Below saturation, the same effect occurs but not as dramatically. If $K_1 > K_2$, the uninhibited form is more strongly bound and inhibition disappears at lower calcium concentrations than when $K_1 = K_2$.

The curves shown in Figure 4 have been calculated using $n = 2$. The number of moles of Ca bound by T during the transition from the inhibited to the noninhibited form (n) is a measure of the cooperativity of the system. When $n = 2$, the steepness of the transition from inhibition to activity is in reasonable agreement with the experimental data. By properly selecting the values of n and K_1/K_2, it is possible to fit the experimental curves. The above calculations indicate that the experimental data are consistent with $K_1 < K_2$.

The binding of calcium by troponin in the actomyosin complex is the same as free troponin only when $K_1/K_2 = 1$ and can be shown by solving eq 4 in terms of the other dissociation constants. When $K_1 \neq K_2$, the binding of calcium by troponin is clearly affected by the interaction with the actomyosin complex.

$$K_4 = \frac{(TA)(Ca^n)}{(TCa_nA)} = K_3\frac{K_2}{K_1} \tag{13}$$

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