A THEORETICAL ANALYSIS OF BINDING TO THE CA^{2+}-SPECIFIC SITES ON TROPONIN INCORPORATED INTO THIN FILAMENTS

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ABSTRACT Recent data on the binding of Ca^{2+} to the specific sites on troponin, alone, in regulated actin, and in regulated actomyosin, as well as data on the Ca^{2+} activation of the actomyosin ATPase (Grabarek, Z., J. Grabarek, P. C. Leavis, and J. Gergely, 1983, *J. Biol. Chem.*, 258:14098–14102.), are analyzed on the basis of a model used previously for qualitative theoretical studies of the Ca^{2+} activation of muscle contraction (Shiner and Solaro, 1982). The data allow and require an extension of the model to consider the effects of tropomyosin explicitly. Three major results of the analysis are at variance with previous investigations. (*a*) A repulsive interaction between tropomyosins; and (*b*) an attractive interaction between actins (or myosin heads attached to actin) are found, whereas others have found or assumed an attractive tropomyosin-tropomyosin interaction and no actin-actin interaction. (*c*) The parameter values found here predict hysteresis under the conditions of the ATPase experiments; no other existing model for the interactions manifest in the Ca^{2+} activation of contraction can predict hysteresis. The prediction is of increased interest in light of experimental reports of hysteresis in the Ca^{2+} activation of isometric force (Ridgeway, E. B., A. M. Gordon, and D. A. Martyn, 1983, *Science (Wash. DC)*, 219:1075–1077; Gordon, A. M., E. B. Ridgeway, and D. A. Martyn, 1984, Plenum Publishing Corp., New York, 553–563; Brandt, P. W., B. Gluck, M. Mini, and C. Cerri, 1985, *J. Mus. Res. Cell Motil.* 6:197–205.).

INTRODUCTION

We have previously presented a model of the Ca^{2+} activation of striated muscle contraction in which we attempted to take into account two essential aspects of the phenomenon in a manner that was simultaneously general and as simple as possible (Shiner and Solaro, 1982). We could not go further than a qualitative analysis at that time because of the lack of appropriate data. Recently, however, Grabarek et al. (1983) have measured the binding of Ca^{2+} to troponin (Tn) alone, incorporated into regulated thin filaments (regulated actin) and incorporated into regulated thin filaments in the presence of myosin and ATP (regulated actomyosin) through fluorescence changes. Under the last conditions they also measured the activation of the ATPase. These data are sufficient to allow us to begin a quantitative analysis of the Ca^{2+} activation of contraction based on the previous model.

The two essential aspects of the Ca^{2+} activation considered in the previous model (Shiner and Solaro, 1982) are: (*a*) given that Ca^{2+} ions activate contraction (Ebashi and Endo, 1968; Weber and Murray, 1973), presumably upon binding to Tn, there must be an interaction between the binding and the contractile event itself—reactions involving actin, myosin, ATP and the products of its hydrolysis; and (*b*) active contraction is a nonequilibrium process driven by the affinity of the ATP hydrolysis catalyzed by (acto-) myosin, so that the simplest condition under which muscle functions is a nonequilibrium stationary state (i.e., a steady state), not thermodynamic equilibrium. Point *a* was taken into account by viewing the thin filament as an (unspecified) lattice of two sorts of sites, Ca^{2+} binding sites (on Tn) and actins, which react with myosin crossbridges and their complexes with the various adenosine nucleotides. Interactions were assumed between nearest neighbor Ca^{2+} binding sites and actins, such that the reaction of myosin with actin is stabilized when a nearest neighbor Ca^{2+} site is occupied and vice versa. For good measure, interactions were also allowed betwen nearest neighbor Ca^{2+} sites and between nearest neighbor actins. Tropomyosin (TM) was not explicitly considered in the original model. Since Grabarek et al. (1983) present Ca^{2+} binding data for Tn alone and for Tn incorporated into thin filaments it is now possible to explicitly consider the role of TM in the various interactions. The extension of the primitive model necessary to take TM into account will be presented here. Point *b* was formulated as a two-state attachment-detachment cycle involving actin and myosin, with the condition on the four pseudo-first-order rate constants of the cycle that they not correspond to thermodynamic equilibrium.

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Although this primitive model is probably the simplest possible which takes into account points (*a*) and (*b*) in any generality, no complete, exact solution is known for such nearest neighbor models for arbitrary lattices even at thermodynamic equilibrium (Bragg and Williams, 1934), much less at nonequilibrium stationary states (Hill, 1977*b*); therefore, solutions were presented in terms of the simplest approximation available, the mean field approximation (Bragg and Williams, 1934). The most interesting of the insights offered by the model (Shiner and Solaro, 1982, 1984) for the discussion of the results obtained here is the prediction of the possibility of hysteresis in the stationary state Ca^{2+} activation. Hysteresis in the Ca^{2+} activation of isometric force in barnacle fibers (Ridgway et al., 1983; Gordon et al., 1984) and in mammalian skeletal muscle (Gordon et al., 1984; Brandt et al., 1985) has now been reported.

Grabarek et al. (1983) present binding data for both the Ca^{2+}-specific sites and the Ca^{2+}-Mg^{2+} sites on Tn. Although on the basis of our previous qualitative results (Shiner and Solaro, 1982) we have argued that the reasoning leading to the conclusion that only the Ca^{2+}-specific sites are responsible for the activation of contraction (Potter and Gergely, 1975; Johnson et al., 1979; Robertson et al., 1981) is not decisive, and although hysteresis in the activation of stationary state force further weakens this reasoning, only binding to the Ca^{2+}-specific sites will be treated here (Fig. 2 *A* of Grabarek et al.). Since the binding data for the Ca^{2+}-Mg^{2+} sites (Fig. 2 *B* of Grabarek et al.) seem, to a first approximation, to be the same in the Ca^{2+} binding subunit of Tn (TnC), in whole Tn, and in Tn incorporated into thin filaments both in the absence and presence of myosin, it seems indeed, again at least to a first approximation, that the Ca^{2+}-Mg^{2+} sites are not involved in the activation of contraction; if binding to these sites is not affected by the presence of TM in thin filaments nor by the reaction of myosin with actin, then conversely binding to these sites affects neither the state of TM nor the reaction of myosin with actin.

The binding of Ca^{2+} to isolated TnC, for which Grabarek et al. (1983) also present data, will also not be treated here. The step from isolated TnC to whole Tn is too large for the Ca^{2+} binding curves from TnC and Tn to be sufficient to determine values for all parameters possibly involved. There are possible binary interactions between all three components of Tn (TnC, TnI, and TnT) as well as additional interactions that might arise in the ternary complex of whole Tn. The treatment necessary for such an analysis when enough data become available would be similar to that given below in going from Tn to regulated actin to regulated actomyosin.

Three discrepancies will be found below between the results here and those of other theoretical analyses. (*a*) A repulsive interaction is found between TM molecules whereas others have found (Wegner, 1979) or assumed (Hill et al., 1980, 1981, 1983; Hill, 1983) an attractive one. (*b*) An attractive interaction is found when myosins are attached to nearest neighbor actins; others have assumed no interaction (Hill et al., 1980, 1981, 1983; Hill, 1983). (*c*) Under certain conditions the parameter values found here predict hysteresis in Ca^{2+} binding to regulated actomyosin and in the Ca^{2+} activation of actomyosin ATPase; no other model for the Ca^{2+} activation known to me can predict hysteresis regardless of the parameter values chosen.

The rest of the paper is organized as follows. In the next section the theory is outlined and the appropriate equations developed. The methods and results of the data analysis are then presented for the four cases: Ca^{2+} binding to (*a*) Tn alone, (*b*) regulated actin and (*c*) regulated actomyosin; and (*d*) Ca^{2+} activation of the ATPase. Discussion of the results follows. A discussion of the prediction of hysteresis and its relation to that seen experimentally in the activation of force is reserved for the Appendix.

THEORY

Since a detailed derivation of the previous model (Shiner and Solaro, 1982) and the generalization necessary to take TM into account explicitly have already been presented (Shiner and Solaro, 1981), the appropriate equations will not be developed here with any rigor. Rather, only plausibility arguments will be presented for the mean field approximation to offer some insight into the significance of the various parameters.

General

A binding isotherm at thermodynamic equilibrium may generally be written in the form

$$\theta = \frac{Qz}{1 + Qz} \Longleftrightarrow \frac{\Delta G}{kT} \equiv -\ln Q = \ln\left[\frac{(1 - \theta)z}{\theta}\right], \qquad (1)$$

where z is the concentration of the ligand being bound, θ the fractional occupation of the binding sites, Q the apparent binding constant, ΔG the change in free energy upon binding, k the Boltzmann constant, and T temperature. If ΔG (and therefore Q) is constant, one says that the system is noncooperative. If, on the other hand, ΔG depends on θ (or z), one speaks of interactions between binding sites.

To study such interactions we picture a cooperative binding system as a lattice of binding sites such that each site has c nearest neighbors (Ising, 1925; Bragg and Williams, 1934; Hill, 1977*a*, *b*; Shiner and Solaro, 1981). When a site is in state A and a nearest neighbor is in state B, where A and B are either o or x, denoting the unoccupied (free) and occupied (ligand-bound) state, respectively, we assume a (free) energy of interaction w^{AB}. Unfortunately, even for this simple picture of interactions, no exact, analytic solution is known for an arbitrary lattice (as specified by c). The simplest approximation, the mean field approximation (Bragg and Williams, 1934; Hill,

1977*b*; Shiner and Solaro, 1981), leads to a linear dependence of ΔG on θ:

$$\Delta G = \hat{u} + c(w^{ox} - w^{oo}) + c(w^{xx} - 2w^{xo} + w^{oo})\theta, \quad (2)$$

where $\hat{u}$ is the free energy of binding to a site subject to no interactions, and $w^{xo} = w^{ox}$. With this form for ΔG, the binding isotherm, Eq. 1, becomes:

$$\ln z = \ln\left(\frac{\theta}{1-\theta}\right) + \frac{u}{kT} + \frac{cw}{kT}\theta, \quad (3)$$

where

$$u \equiv \hat{u} + c(w^{ox} - w^{oo})$$

and

$$w \equiv w^{xx} - 2w^{xo} + w^{oo}.$$

The above has been for a lattice of a single class of sites. If the lattice has more than one class of sites, Eq. 3 can easily be generalized to

$$\ln z_i = \ln\left(\frac{\theta_i}{1-\theta_i}\right) + \frac{\hat{u}_i}{kT} + \sum_j \frac{\Delta_{ij}}{kt} + \sum_j \frac{c_{ij}w_{ij}}{kT}\theta_j;$$

$$\Delta_{ij} \equiv c_{ij}(w_{ij}^{xo} - w_{ij}^{oo});$$

$$w_{ij} \equiv w_{ji} \equiv w_{ij}^{xx} - w_{ij}^{xo} - w_{ij}^{ox} + w_{ij}^{oo}. \quad (4)$$

w_{ij}^{AB} is the energy of interaction when a class i site is in state A and a nearest neighbor class j site is in state B, and each site of class i has c_{ij} nearest neighbors of class j. ($w_{ij}^{AB} = w_{ji}^{BA}$). θ_i is the fractional occupation of class i sites, and z_i, the corresponding free ligand concentration. Of course, there is an Eq. 4 for each class of sites. Note also that if there are M_i sites of class i, then the total number of ij nearest neighbor pairs, $i \neq j$, counting from the i sites is $c_{ij}M_i$. Counting from the j sites yields $c_{ji}M_j$; thus we have the conservation relation

$$c_{ij}M_i = c_{ji}M_j. \quad (5)$$

Since only the quantities w_{ij} play a role in the dependence of the free energies of binding on the θ_i's, they will be referred to simply as (net or effective) ij interaction energies.

Thin Filament

We picture the thin filament as a lattice of three classes of sites: (*a*) the Ca^{2+}-specific sites on Tn; (*b*) tropomyosin molecules, which can be in one of two states, "off" or "on"; (*c*) actins, to which myosin heads can attach. A possible structure is shown in Fig. 1. We assume that the Ca^{2+}-specific sites do not interact directly with the actins, even though the inhibitory subunit of Tn does bind to actin (Potter and Gergely, 1974). The Ca^{2+} binding isotherm

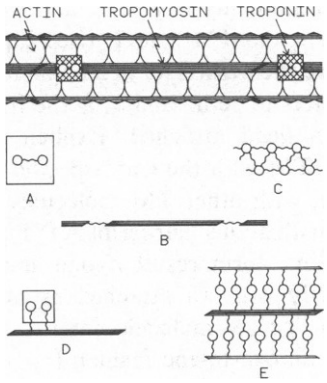

FIGURE 1 A schematic illustration of a possible interaction structure for the thin filament. To obtain the picture in the *upper* part of the figure, the thin filament helix is first unwound. Then one TM strand is cut down the middle and the entire structure unfolded. The Tn molecules associated with the cut TM strand are not shown. (Compare Fig. 1 of Adelstein and Eisenberg, 1980.) In the *lower* part of the figure possibilities for the individual interactions are illustrated in an exploded view; the interactions are shown as squiggly lines. (*A*) Each Ca^{2+}-specific site interacts with its partner within the same Tn molecule; $c_{CC} = 1$. (*B*) The TM molecules interact end-to-end along a TM strand; $c_{TT} = 2$. (*C*) Each actin interacts with four nearest neighbor actins ($c_{AA} = 4$); the interactions between nearest neighbors in the same strand could be different than those between nearest neighbors in different strands, although this is not considered here. (*D*) Each Ca^{2+}-specific site of a Tn molecule interacts with the TM molecule to which the Tn belongs; $c_{CT} = 1$; $c_{TC} = 2$. (*E*) Each actin interacts with both TM strands, and each TM therefore with 14 actins; $c_{AT} = 2$; $c_{TA} = 14$.

then becomes, from Eq. 4,

$$\ln z_C = \ln\left(\frac{\theta_C}{1-\theta_C}\right) + \frac{u_C^o}{kT} + \frac{\Delta_{CT}}{kT} + \frac{w_{CC}}{kT}\theta_C + \frac{c_{CT}w_{CT}}{kT}\theta_T, \quad (6a)$$

where $u_C^o = \hat{u}_C + \Delta_{CC}$, the subscript C denotes Ca^{2+}-specific site, the subscript T denotes tropomyosin, and θ_T is the fraction of TM molecules in the on state. We have assumed that interactions between Ca^{2+}-specific sites occur only within a given Tn molecule and have already taken into account in Eq. 6a that $c_{CC} = 1$, since there are only two Ca^{2+}-specific sites on a given Tn molecule (Fig. 1 *A*). c_{CT}, the number of TM molecules with which a given Ca^{2+}-specific site interacts, is also presumably 1, but we leave it unspecified, since only the quantity $c_{CT}w_{CT}/kT$ plays a role anyway (Fig. 1 *D*).

In the equation for TM analogous to Eq. 6a the term z_T on the left-hand side of Eq. 4 vanishes, since the equation describes not the binding of TM but a conformational change

$$o = \ln\left(\frac{\theta_T}{1-\theta_T}\right) + \frac{u_T}{kT} + \frac{2c_{CT}w_{CT}}{kT}\theta_C + \frac{c_{TT}w_{TT}}{kT}\theta_T + \frac{7c_{AT}w_{AT}}{kT}\theta_A, \quad (6b)$$

where $u_T = \hat{u}_T + \Delta_{TC} + \Delta_{TT} + \Delta_{TA}$. Here we have taken into account that $c_{TC} = 2c_{CT}$, since the stoichiometry of Tn

to TM in thin filaments is 1 to 1, and therefore that of Ca^{2+}-specific sites to TM 2 to 1. (See Eq. 5.) Similarly, $c_{TA} = 7c_{AT}$ since the actin-TM stoichiometry is 7 to 1. The subscript A refers to actin, and θ_A is the fraction of actins with a myosin head attached. Explicit in Eq. 6b are interactions of TM with the Ca^{2+}-specific sites (subscript CT; Fig. 1 *D*), with other TM molecules (subscript TT; Fig. 1 *B*) and with actins (subscript AT; Fig. 1 *E*). (Equations of the same form result if one assumes that TM interacts directly with an attached myosin head rather than an actin.) The TM molecules interact with each other presumably in an end-to-end fashion ($c_{TT} = 2$), but again we leave c_{TT} unspecified. It is usually assumed that each actin interacts only with one TM molecule ($c_{AT} = 1$) (Hill et al., 1980, 1981, 1983; Hill, 1983), but it is easy to imagine that each TM molecule interacts with both strands of the actin double helix (see also Fig. 1 of Adelstein and Eisenberg, 1980), and that each actin is therefore influenced by two TM molecules ($c_{AT} = 2$).

Similarly, for the attachment of myosin heads to actin we have

$$\ln z_M = \ln\left(\frac{\theta_A}{1-\theta_A}\right) + \frac{u_A}{kT} + \frac{c_{AT}w_{AT}}{kT}\theta_T + \frac{c_{AA}w_{AA}}{kT}\theta_A, \quad (6c)$$

where $u_A = \hat{u}_A + \Delta_{AT} + \Delta_{AA}$. z_M is the concentration of free myosin. The actin-actin (AA) interaction (Fig. 1 *C*) has been neglected by the Bethesda school (Hill et al., 1980, 1981, 1983; Hill, 1983) but has been included in the treatment by Balazs and Epstein (1983) of the binding of subfragment 1 (S1) to regulated actin. The latter authors assumed $c_{AA} = 2$, i.e. linear interactions along an actin strand of the thin filament. However, since F-actin is a double helix, comprised of two strands, each actin actually has four nearest neighbors (see also Fig. 1 of Adelstein and Eisenberg, 1980 again), although all nearest neighbors might not be equivalent. Therefore, one could just as easily take $c_{AA} = 4$. (Note that these interactions might just as well be called interactions between S1 binding sites and/or occur between attached myosin heads rather than between actins; this would not change the form of Eq. 6c.)

Additional assumptions are implicit in Eq. 6c. The most important is that the reaction of actin and myosin in the presence of ATP (the conditions of the binding experiments to regulated actomyosin) can be described adequately by a two-state cross-bridge cycle, in which an actin either has a myosin head attached to it or not. (See, e.g., Fig. 1 *B* of Shiner and Solaro, 1982, or Fig. 7 of Hill, 1983.) Such two-state cycles have proven useful for qualitative analyses and semiquantitative evaluations (Huxley, 1957; Shiner and Solaro, 1982; Hill, 1983). Nonetheless, a two-state cycle must be viewed as a severe approximation since steady state and transient studies of the actomyosin ATPase have required as many as ten states for their explanation. (See Adelstein and Eisenberg, 1980 for a review.) Less severe approximations use cross-bridge cycles with three or four states (Eisenberg and Hill, 1978; Eisenberg et al., 1980; Hill et al., 1981) and seem to be necessary for hard quantitative analyses of the actin-myosin-ATP reactions. However, since the subject at hand here is Ca^{2+} binding (and Ca^{2+} activation of the actomyosin ATPase) it may be hoped that a two-state cycle is sufficient. Moreover, Eqs. 6a–c already contain nine parameters to be evaluated from the binding data, and the inclusion of the additional parameters that would be required for cross-bridge cycles with more than two states hardly seems justified.

The second assumption implicit in Eq. 6c is the quasi-equilibrium assumption (Hill, 1977*a*, *b*; Shiner and Solaro, 1981). Although this equation is supposed to represent a nonequilibrium stationary state it has an equilibrium form. In general the interactions with actin (or myosin) will affect the two pathways of the two-state cross-bridge cycle differently, and the isotherm for the attachment of myosin to actin will not take an equilibrium form. If, however, both pathways are affected equally, an equilibrium form does result, although apparent equilibrium constants are then not true equilibrium constants but rather analogous to Michaelis constants; i.e., u_A is not a true binding energy but of the form $kT\ln(K_m)$, where K_m is a Michaelis-like constant. In light of the simplifications and approximations already made, the quasiequilibrium assumption would seem to do no further harm, particularly since its relaxation would again require the introduction of additional parameters.

The final approximation in Eq. 6c is that the two-headed nature of myosin can be neglected. Hill (1978) has shown how to take this feature into account, and there are experimental indications of how it may manifest itself (Shukla et al., 1984). Nonetheless, it will be assumed here either that only one head can attach to actin at a time (Chen and Reisler, 1984) or that both heads can attach independently. In the latter case the effective concentration of myosin is twice that in the former. In addition to the justifications already mentioned for the approximations of a two-state cycle and the quasiequilibrium assumption, the results of Eaton (1976) indicate that this approximation is reasonable. She found that plots of the induction of TM binding to F-actin against the molar ratio of myosin heads to actin monomers were identical regardless of whether the heads came from S1 (one-headed) or heavy meromyosin (two-headed).

Ca^{2+} Binding to TN Alone

For the binding to the Ca^{2+}-specific sites of Tn alone we have, of course, only Eq. 6a. In addition, the Δ_{CT} and w_{CT} terms vanish, since they represent the effects of interactions with TM in the thin filament

$$\ln z_C = \ln\left(\frac{\theta_C}{1-\theta_C}\right) + \frac{u_C^o}{kT} + \frac{w_{CC}}{kT}\theta_C. \quad (7)$$

Ca^{2+} Binding to Regulated Actin

Here we have both the Ca^{2+} and TM eqs. 6a, b, with $\theta_A = 0$ in Eq. 6b, since myosin is absent

$$\ln z_C = \ln\left(\frac{\theta_C}{1-\theta_C}\right) + \frac{u_C^o}{kT} + \frac{\Delta_{CT}}{kT} + \frac{w_{CC}}{kT}\theta_C + \frac{c_{CT}w_{CT}}{kT}\theta_T \quad (8a)$$

$$o = \ln\left(\frac{\theta_T}{1-\theta_T}\right) + \frac{u_T}{kT} + \frac{2c_{CT}w_{CT}}{kT}\theta_C + \frac{c_{TT}w_{TT}}{kT}\theta_T. \quad (8b)$$

Ca^{2+} Binding to Regulated Actomyosin

All three Eqs. 6 are necessary in this case, but there is a slight complication. The concentration of free myosin z_M was not held constant in these experiments but rather the total myosin concentration $\mathcal{M}$. The free concentration can easily be calculated, however. Since θ_A is the fraction of actins with a myosin head attached, $\mathcal{A}\theta_A$ is the concentration of attached myosin heads, where $\mathcal{A}$ is the total concentration of actin. The concentration of free myosin is then

$$z_M = \mathcal{M} - \mathcal{A}\theta_A = \mathcal{A}\left(\frac{\mathcal{M}}{\mathcal{A}} - \theta_A\right) = \mathcal{A}(r - \theta_A); \quad r \equiv \frac{\mathcal{M}}{\mathcal{A}} \quad (9)$$

For the conditions of these experiments as given in the legend to Fig. 2 of Grabarek et al. (1983). $r = 0.4375$ assuming one-headed attachment of myosin to actin. If both heads can attach, we must multiply by two: $r = 0.875$. Putting Eq. 9 into Eq. 6c, Eqs. 6a–c for Ca^{2+} binding to regulated actomyosin become

$$\ln z_C = \ln\left(\frac{\theta_C}{1-\theta_C}\right) + \frac{u_C^o}{kT} + \frac{\Delta_{CT}}{kT} + \frac{w_{CC}}{kT}\theta_C + \frac{c_{CT}w_{CT}}{kT}\theta_T \quad (10a)$$

$$o = \ln\left(\frac{\theta_T}{1-\theta_T}\right) + \frac{u_T}{kT} + \frac{2c_{CT}w_{CT}}{kT}\theta_C + \frac{c_{TT}w_{TT}}{kT}\theta_T + \frac{7c_{AT}w_{AT}}{kT}\theta_A \quad (10b)$$

$$o = \ln\left[\frac{\theta_A}{(1-\theta_A)(r-\theta_A)}\right] + \frac{u'_A}{kT} + \frac{c_{AT}w_{AT}}{kT}\theta_T + \frac{c_{AA}w_{AA}}{kT}\theta_A, \quad (10c)$$

where $\exp(u'_A/kT) = [\exp(u_A/kT)]/a$.

ATPase

In the presence of myosin and ATP the activation of the ATPase was also measured. Regardless of whether the ATPase is considered reversible or irreversible, it can easily be shown (Shiner and Solaro, 1981) (see also Eq. 3 of Shiner & Solaro, 1982) that at the stationary state it takes the form

$$J = \kappa e^{(f_{AA}c_{AA}w_{AA}/kT)\theta_A} e^{f_{AT}c_{AT}w_{AT}\theta_T/kT}\theta_A, \quad (11a)$$

where f_{AA} and f_{AT} are constants according to the formulation of Hill (1977*a*, *b*), and θ_A and θ_T are given by Eqs. 10a–c. The proportionality constant κ plays no role here since only the relative activation j of the ATPase was measured

$$j \equiv (J - J_0)/(J^* - J_0) \quad (11b)$$

where $J_0 = \lim_{z\to 0} J$ and $J^* = \lim_{z\to\infty} J$.

The ratio of myosin to actin concentrations r in Eq. 10c is slightly different for these experiments than for the Ca^{2+} binding experiments. Again using the values for $\mathcal{M}$ and $\mathcal{A}$ given in Grabarek et al. (1983), we calculate $r = 0.5$ for one-headed attachment and $r = 1.0$ for two-headed attachment.

METHODS AND RESULTS

In all three cases of Ca^{2+} binding parameters were evaluated by minimizing the sum of the errors squared in θ_C as a function of z_C. For the ATPase the same was done for j as a function of θ_T and θ_A. For none of these cases can the parameter values that minimize the sum of the errors squared be found analytically. Therefore, the problem was attacked via an iterative procedure with convergence assumed when the relative change in all parameters was $<10^{-6}$.

θ_C (and θ_T and θ_A where appropriate) can also not be calculated analytically as a function of z_C from the equations of the previous section. These calculations were also carried out iteratively. Since the equations may predict a phase transition, which may manifest itself as hysteresis (see below), it was assumed that the data correspond to the ascending limb of the hysteresis loop if this is predicted. Therefore, iterations were always started with initial values for all θ less than their stationary state (or equilibrium) values, and step size was varied so that all $d\theta/dt$ always remained >0. The convergence criterion (relative change in all θ) was again taken as 10^{-6}.

The parameter values found after convergence are given in Table I; the corresponding Ca^{2+} binding curves along with the data from Grabarek et al. (1983) are shown in Figs. 2 and 3. u_C^o/kT and w_{CC}/kT were found from Eq. 7 and the data for binding to the Ca^{2+}-specific sites for Tn alone. Given these two values, Δ_{CT}/kT, $c_{CT}w_{CT}/kT$, u_T/kT and $c_{TT}w_{TT}/kT$ were then obtained from the data for binding to regulated actin and eqs. 8a–b. There was nothing remarkable about the choice of initial values used to begin the iterative least squares procedure for these two cases.

TABLE I

Parameter estimates from least-squares analyses	
$u_C^o = -14.423$	$w_{CC}/kT = -0.178$
$\Delta_{CT}/kT = -0.203$	$c_{CT}w_{CT}/kT = -2.72$
$u_T/kT = 3.56$	$c_{TT}w_{TT}/kT = 2.18$
Two-headed myosin attachment	
$u'_A/kT = 2.66$	$u_A/kT = -10.38$
$c_{AT}w_{AT}/kT = -0.881$	$c_{AA}w_{AA}/kT = -5.58$
$f_{AA} = 0.525$	$f_{AT} = -0.174$
One-headed myosin attachment	
$u'_A/kT = 1.95$	$u_A/kT = -10.40$
$c_{AT}w_{AT}/kT = -1.10$	$c_{AA}w_{AA}/kT = -5.69$
$f_{AA} = 1.76$	$f_{AT} = -1.37$

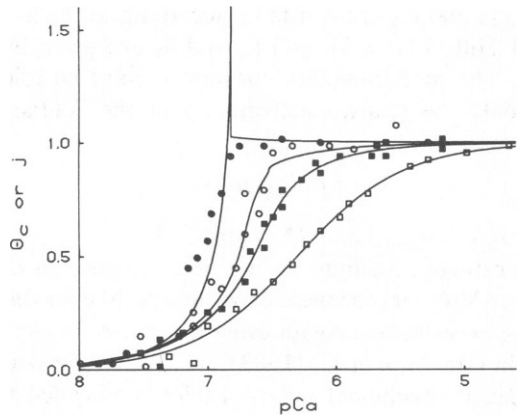

FIGURE 2 Data from Grabarek et al. (1983) for the binding of Ca^{2+} to the Ca^{2+}-specific sites on Tn for Tn alone (□), regulated actin (■) and regulated actomyosin (○), along with the activation of actomyosin ATPase (●). The curves were calculated for these four cases from Eqs. 7, 8, 10, and 11, in this order, with the parameter values obtained in this work. For Ca^{2+} binding to regulated actomyosin and the activation of ATPase it was assumed that both heads of a myosin molecule can bind to actin independently. (r = 0.875 for Ca^{2+} binding and 1.0 for ATPase). Only the ascending branch of the hysteresis loop for the ATPase is shown.

The data for Ca^{2+} binding to regulated actomyosin were analyzed with the parameters already found and eqs. 10a–c to find $c_{AT}w_{AT}/kT$, $c_{AA}w_{AA}/kT$, and u'_A/kT, both for the assumption of one-headed myosin attachment and that of two-headed attachment. The iterative least-squares procedure in this case was sensitive to the initial guesses for these parameters for two-headed attachment and converged only for a choice of initial values that predicted hysteresis in the Ca^{2+} binding (see below). Note, however, that no hysteresis is predicted for the final parameter values found after convergence (Table I) for the conditions of the binding experiments (Figs. 2 and 3).

For the analysis of the ATPase, θ_T and θ_A were calculated for each data point from the parameters previously determined, and $f_{AA}c_{AA}w_{AA}/kT$ and $f_{AT}c_{AT}w_{AT}/kT$ determined from j as a function of θ_T and θ_A (Eq. 11). The calculated curves are again shown in Figs. 2 and 3 along with the data. Note that for the assumption of two-headed myosin attachment, hysteresis is predicted under the conditions of these experiments (r = 1; see Fig. 4); only the ascending branch is shown in Fig. 2. In this case it was also necessary to constrain the least-squares procedure so that an activation of the ATPase by Ca^{2+} was obtained; otherwise, the procedure converged to parameter values that predicted an inhibition of the ATPase by Ca^{2+}.

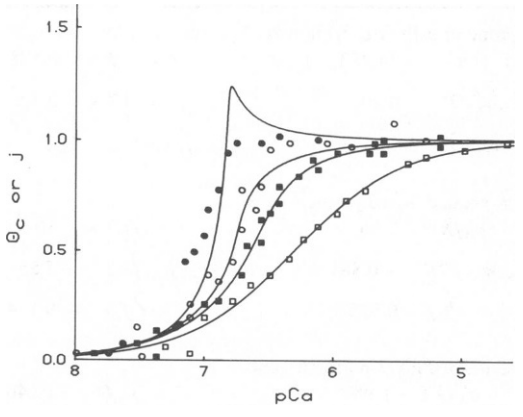

FIGURE 3 Same as Fig. 2, except that it was assumed that only one head of a myosin molecule can attach to actin at a time. (r = 0.4375 for Ca^{2+} binding to regulated actomyosin; r = 0.5 for the ATPase.) There is no hysteresis in the ATPase curve in this figure.

DISCUSSION

General

It should be kept in mind throughout the discussion that Grabarek et al. (1983) did not measure Ca^{2+} binding directly, but rather fluorescence changes induced by the binding. Grabarek et al. (1983) and Grabarek and Gergely (1983) themselves discuss in some detail how the relationship between fluorescence and binding would affect their results. Of course, this relationship also affects the results of the analysis here.

Ca^{2+} Binding to Tn Alone

The parameter values found in this case, $u_C^0/kT = -14.423$ and $w_{CC}/kT = -0.178$, correspond to a Ca^{2+} concentration z_C at half-maximal binding ($\theta_C = \frac{1}{2}$) of 4.98×10^{-7} M or $pCa_{1/2} = 6.30$. [$pCa = -\log_{10}(z_C/M)$.] This agrees well with the results of Grabarek et al. (1983) ($pCa_{1/2} = 6.3$) and of Potter and Gergely (1975) ($pCa_{1/2} = 6.7$). As pointed out by Grabarek and Gergely (1983), the low strength of interaction found here ($w_{CC}/kT = -0.178$) may not be due to actual interactions but rather may be an artifact due to their having measured Ca^{2+} binding through fluorescence changes.

It is of interest here to compare the results for the mean field approximation to those for exact calculations, which can easily be carried out in this case since there are only two Ca^{2+}-specific sites on a given Tn molecule. The exact binding isotherm can be written

$$\theta_C = \frac{K_0 z_C + K_I K_0^2 z_C^2}{1 + 2K_0 z_C + K_I K_0^2 z_C^2},$$

where $-\ln K_0$ and $-\ln K_I$ are the quantities analogous to u_C^0/kT and w_{CC}/kT in the approximation. To compare the exact quantities with those of the approximation, we calculate the values of K_0 and K_I for the above equation, which give the same z_C and slope $(\partial\theta_C/\partial \ln z_C)$ at $\theta_C = \frac{1}{2}$ as do the fitted parameters in Eq. 7. We find $-\ln K_0 = -14.149$ and $-\ln K_I = -0.186$; these are within 0.3% and 5%, respectively, of the values from the approximation. Thus, the approximation results in good estimates here. However, this would be quantitatively less the case for stronger interactions.

This comparison points out one important effect of the approximation, namely that it underestimates the strength of interactions; i.e. $|w_{CC}/kT| < |-\ln K_I|$. In other words, the mean field approximation overestimates the effect of a given interaction energy, particularly as manifest in the slope of a binding curve. This property of the approximation may have implications for the cases discussed below.

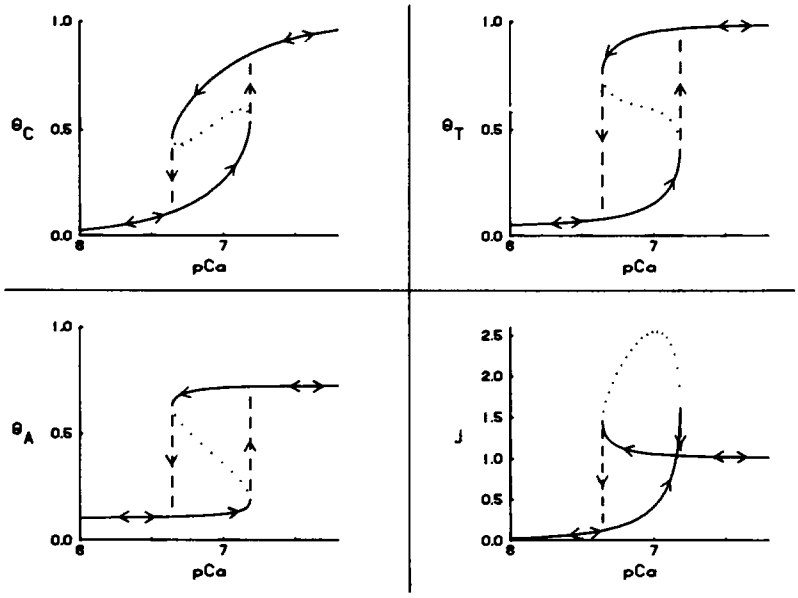

FIGURE 4 The hysteresis predicted for the conditions of the ATPase measurements with the assumption of two-headed myosin attachment. The solid portions of the curves are stable, and the dotted portions unstable. The vertical dashed lines represent the jumps from one branch of the hysteresis loop to the other.

Ca^{2+} Binding to Regulated Actin

The simple incorporation of Tn into the thin filament leads to a slight decrease in the free energy of binding to a single, Ca^{2+}-specific site even if all TM molecules would be in the off state; $\Delta_{CT}/kT = -0.203$. The net Ca^{2+}-TM interaction energy that arises when a Ca^{2+}-specific site is occupied and a nearest neighbor TM molecule is in the on state, compared to that when TM is in the off state, represents a strong cooperative interaction; $c_{CT}w_{CT}/kT = -2.72$. The equilibrium constant for the transition off to on for a TM molecule when all nearest neighbor TM's are in the off state and all nearest neighbor Ca^{2+}-specific sites are unoccupied is $\exp(-u_T/kT) = \exp(-3.56) = 0.0284$. Furthermore, there is a not insignificant repulsive interaction ($c_{TT}w_{TT}/kT = 2.18$) between nearest neighbor TM molecules in the same state. Upon binding of Ca^{2+} to Tn, the fraction of TM's in the on state increases from 0.0262 in the absence of Ca^{2+} to 0.626 at saturating Ca^{2+}.

The data of Grabarek et al. (1983) and thus the results of the analysis here are at odds with those of two other studies. Zot et al. (1983) (using rabbit skeletal muscle proteins), and Wnuk et al. (1984) (using regulated actin reconstituted from rabbit actin and the TM-Tn complex from crayfish) found that the $pCa_{1/2}$ for Ca^{2+} binding to regulated actin was higher than that for pure Tn, whereas Grabarek et al. found the opposite. There are two obvious possible explanations for these differences. The first is the difference between binding and fluorescence, as mentioned above and discussed by Grabarek et al. and Grabarek and Gergely (1983) themselves. The second possible explanation is differences in experimental conditions (or species differences in the case of Wnuk et al.). This possibility is made more attractive when one notes that a change in a single parameter would yield a $pCa_{1/2}$ shift in the same direction found by Zot et al. and Wnuk et al. The Ca^{2+} binding curve to regulated actin can be shifted at will by changing Δ_{CT} (Eq. 8a). A shift in the direction opposite to that found by Grabarek et al. and here would be found if Δ_{CT} were >0 instead of <0. Since Δ_{CT} is essentially a free energy, changes in experimental conditions could easily change its sign, particularly since the value found here is not very different from zero.

Another serious discrepancy exists between the results here and those of the studies of S1 binding by the Bethesda school (Hill et al., 1980, 1983), where it is found that the TM-TM interaction is attractive (<0). Let us assume for the moment that $c_{TT}w_{TT}/kT$ should be attractive and ask how the analysis here could result in a repulsive interaction. As mentioned above in connection with the case of binding to Tn alone, the mean field approximation overestimates the effect of an interaction on the slope of the binding curve. Assuming that a large negative value of $c_{CT}w_{CT}/kT$ is necessary to account for the leftward shift of the Ca^{2+} binding curve on incorporation of Tn into regulated thin filaments (Figs. 2 and 3), this value would lead to a slope that is too steep; this effect could then be compensated by a repulsive TM-TM interaction, which tends to decrease the slope. On the other hand, the factor Δ_{CT}/kT in Eq. 8a could alone account for the leftward shift, so that the shift does not necessitate the large negative value of $c_{CT}w_{CT}/kT$; rather, $c_{CT}w_{CT}/kT$ and $c_{TT}w_{TT}/kT$ are together responsible for the slope of the binding curve.

Thus we are left with the possibility that $c_{TT}w_{TT}/kT$ is truly repulsive (>0). How can we then account for the

attractive TM-TM interaction of the Bethesda school? In their work on the binding of S1 to regulated actin (Hill et al., 1980, 1983), on regulated actomyosin ATPase (Hill et al., 1981), and on the Ca^{2+} activation of muscle contraction (Hill, 1983) they have considered particular models, usually and most importantly that each Ca^{2+} binding site interacts with one TM, as does an actin, and that each TM interacts with seven actins and the relevant Ca^{2+} binding sites of the adjacent Tn. [i.e., $c_{CT} = 1$, $c_{AT} = 1$, $c_{TA} = 7$, $c_{TC} = 2$ (for only the Ca^{2+}-specific sites, for example).] Furthermore, direct actin-actin (or bound S1-S1) interactions are neglected. To account for the steepness of the S1 binding curves they then have to include attractive end-to-end TM-TM interactions. This is apparently in accord with the results of Wegner (1979) on TM binding to F-actin. His analysis via the McGhee-von Hippel (1974) model also indicated attractive TM-TM interactions.

Nagashima and Asakura (1982) noted, however, that interaction topologies other than those used by the Bethesda school are possible; for example each TM might influence fourteen actins ($c_{TA} = 14$), as is illustrated in Fig. 1. Again using a particular model with $c_{TA} = 16$ and $c_{AT} = 1$ (which is actually not allowed, since then $c_{AT}M_A \neq c_{TA}M_T$; see Eq. 5), Nagashima and Asakura could in fact adequately reproduce their data without any TM-TM interaction at all. Experimental evidence that end-to-end TM-TM interactions may not be significant is also available. Walsh et al. (1984) found that various measures of the cooperativity of the Ca^{2+} activation of acto-S1 ATPase were not significantly different regardless of whether regulated acto-S1 was prepared from whole tropomyosin or from a nonpolymerizable TM obtained by removal of 11 amino acid residues from the carboxy-terminal of TM. However, in these studies the molar ratio of total S1 to total actin was only 1 to 100, which would tend to diminish the manifestation of TM end-to-end interactions since the binding of S1 to actin presumably favors the on state of TM. Furthermore, in an earlier similar study, Tawada et al. (1975) found that the replacement of TM with nonpolymerizable TM leads to a significant decrease of the cooperativity in the Ca^{2+} activation of the superprecipitation of regulated actomyosin.

We also find a strong attractive actin-actin interaction in regulated actomyosin here, as discussed below. Balazs and Epstein (1983) also used such attractive interactions to analyze the kinetics of the binding of S1 to regulated actin. The Bethesda school neglects these interactions, however; since they are attractive, and therefore tend to increase the slope of the binding curves, it could be that their neglect forces the result of an attractive TM-TM interaction when it is actually repulsive. Incidentally, although Balazs and Epstein do not explicitly consider TM-TM interactions, they found a net repulsive interaction when S1 was bound to nearest neighbor actins that "belong" to different TM molecules. This finding could also reflect a repulsive TM-TM interaction.

We must still reconcile Wegner's (1979) result with the one obtained here. One should recognize that two different TM-TM interactions may be involved. In Wegner's TM binding experiments the two states of TM are (*a*) free in solution and (*b*) bound to the thin filament. In the S1 binding experiments (Hill et al., 1980, 1983) or the Ca^{2+} binding experiments treated here, however, it is two different states of TM incorporated in the thin filament that are involved, an off and an on state. Nothing excludes the possibility that the interactions in the two cases are different—a net attractive interaction between bound TM molecules, presumably mostly in the off state, in comparison with free TM in Wegner's experiments, but a net repulsive interaction between TM's in the on state in comparison with the off state when TM is already incorporated in the thin filament, as is the case here.

TM-TM interactions seem to be indicated. However, a determination of whether they are repulsive or attractive would seem to have to await further study.

Ca^{2+} Binding to Regulated Actomyosin

In this case the results were surprisingly similar regardless of whether only one ($r = 0.4375$) or both ($r = 0.875$) heads of myosin were assumed to be able to attach to actin (Table I). The curve drawn with parameters for two-headed attachment (Fig. 2) seems to be a slightly better representation of the data than that drawn with the parameters for one-headed attachment (Fig. 3) though. With the actin concentration of 0.2 mg/ml used in these experiments (Grabarek et al., 1983), the values for u'_A/kT give apparent equilibrium constants for the attachment of myosin to actin when no myosins are attached ($\theta_A = 0$) and all TM molecules are in the off state ($\theta_T = 0$) of $\exp(-u_A/kT) = 3.27 \times 10^4\ M^{-1}$ for one-headed attachment and $\exp(-u_A/kT) = 1.61 \times 10^4\ M^{-1}$ for two-headed attachment. The latter value must be multiplied by 2 for reference to the myosin concentration, since it is actually the equilibrium constant for the attachment of myosin heads; this yields $3.22 \times 10^4\ M^{-1}$, almost the same value as for one-headed attachment.

Note that no hysteresis is predicted by these values in the Ca^{2+} binding curves (Figs. 2 and 3) although the initial guesses chosen to begin the least-squares iteration for the assumption of two-headed myosin attachment were chosen so as to show hysteresis. Hysteresis (or a phase transition) would appear, however, if z_C was held constant and the concentration of free myosin z_M was varied. If we assume that the incorporation of TM into the thin filament does not change the actin-actin interactions (this, however, may not be the case; see below) and that $\Delta_{AT} = 0$, we may use the present parameter values in Eq. 6c with $\theta_T = 0$ for the binding of myosin to F-actin. If the binding follows the ascending branch of the hysteresis loop, this equation yields a myosin concentration for half-maximal saturation of the actin monomers in F-actin with myosin heads of 2.46×10^{-6} M for one-headed binding or 2.57×10^{-6} M

for two-headed binding. If the binding instead splits the difference in the two phases and thus follows a Maxwell construction (Huang, 1963), the corresponding values are 1.78×10^{-6} M and 1.91×10^{-6} M, respectively. Given the assumptions and approximations used to obtain Eq. 6c these values compare well with those measured by Greene (1982). She found values of 4.55×10^{-6} M in the presence of ADP and $<10^{-6}$ M in its absence for the binding of myosin and heavy meromyosin to F-actin.

The finding of a strong attractive actin-actin interaction ($c_{AA}w_{AA}/kT < 0$) here is, however, again at variance with the work of the Bethesda school (Hill et al., 1980, 1981, 1983; Hill, 1983). Experimentally they have found no evidence for such interactions in the binding of one-headed (Greene and Eisenberg, 1980) or two-headed (Greene, 1982) species of myosin to F-actin. Hence, in their theoretical work on regulated actin they have neglected the possibility of actin-actin interactions. However, the lack of these interactions in F-actin does not necessarily imply their lack in regulated actin. In a manner similar to that in which it was argued above that the TM-TM interactions appropriate for Ca^{2+} binding may actually be repulsive, it can be argued here that the states of actin in regulated thin filaments, both when a myosin head is attached and when not, are different than the corresponding states in F-actin, so that actin-actin interactions only appear in regulated actin. (Note that the occurrence of high levels of cooperativity in regulated actin do not necessarily imply actin-actin interactions—the cooperativity could be due solely to the actin-TM interaction in principle.) Additionally, as mentioned above, Balazs and Epstein (1983) have successfully reproduced the kinetics of S1 binding to regulated actin by postulating just this sort of interaction.

It must be mentioned, however, that the result here of a strong attractive actin-actin interaction may be an artifact resulting from the assumption of a two-state actomyosin-ATP cycle, the mean field approximation, or the procedures used to estimate the parameters. Nothing much can be said about the assumption of a two-state cycle that has not already been mentioned under the Theory section; it is simply inadequate for an explanation of the reactions of actin, myosin, and ATP (Adelstein and Eisenberg, 1980). Observations that Ca^{2+} affects the binding of different S1-nucleotide species to regulated actin differently (Adelstein and Eisenberg, 1980; Greene and Eisenberg, 1980; Chalovich et al., 1981) only emphasize the inadequacy. Only the possibility that the cycle itself may not be decisive for the finding of a strong attractive actin-actin interaction could weaken this objection. Similar comments apply to the quasiequilibrium assumption to the two-state cycle. It is difficult to rule out the possibility of artifacts due to the mean field approximation other than to note that it is a good qualitative approximation. To attempt to eliminate artifacts due to fitting procedures, an unbiased nonlinear least-squares routine was used. Nonetheless, the choices of initial parameter guesses may have forced convergence to a local minimum in the sum of the errors squared; there may be another, deeper minimum with an insignificant actin-actin interaction. In summary, the question of actin-actin interactions in regulated thin filaments remains open.

ATPase

The theoretical curves in Figs. 2 and 3 give a fair representation of the data, although as for the case of Ca^{2+} binding to regulated actomyosin the assumption of two-headed myosin attachment (Fig. 2) seems to give better results than the assumption of one-headed attachment (Fig. 3). Both curves predict an inhibition of the ATPase at high concentrations of Ca^{2+}, however. (The hysteresis in Fig. 2, of which only the ascending branch is shown, will be handled in the Appendix.) To my knowledge such an inhibition has not been discussed in the literature, but it is apparent in some studies on skeletal actomyosin (Walsh et al., 1984) and cardiac myofibrils (Solaro and Shiner, 1976). The inhibition arises because of conflicting effects of the interactions on the ATPase (Eq. 11a). As z_C increases, θ_T and θ_A increase along with Ca^{2+} binding (θ_C) since w_{CT} and w_{AT} are both < 0. Thus, Ca^{2+} activates the ATPase through the factor θ_A in Eq. 11a. The factor $\exp[(f_{AT}c_{AT}w_{AT}\theta_T)/(kT)]$ also increases with θ_C (and θ_T), since both f_{AT} and w_{AT} are <0. However, as θ_C, θ_T, and θ_A increase, $\exp[(f_{AA}c_{AA}w_{AA}\theta_A)/(kT)]$ decreases since $w_{AA} < 0$ but $f_{AA} > 0$. As Ca^{2+} first starts to bind, the activating factors in Eq. 11a are more important than the inhibitory ones. However, as the activation increases, the influence of the inhibitory factor increases until it finally overcomes the activating effects, and one sees inhibition at high levels of Ca^{2+} and activation.

The degree of activation predicted by the parameter values obtained here is also too low. $J^*/J_0 = 1.49$ for one-headed attachment and 1.31 for two-headed attachment; much higher values are seen experimentally.

The weakness of these results for the ATPase is not unexpected, since its treatment is the least well founded part of this work. The inadequacies of a two-state cycle for the actin-myosin-ATP reactions as discussed above are probably even more decisive for the ATPase than for the attachment of myosin to actin.

APPENDIX

Hysteresis in the Ca^{2+} Activation of Contraction?

Hysteresis is predicted by the parameter values found here for the assumption of two-headed myosin attachment under the conditions of the ATPase experiments ($r = 1$). The complete curves for all variables, not just the ascending branch as for the ATPase in Fig. 2, are shown in Fig. 4. The only difference between the Ca^{2+} binding curve for regulated actomyosin in Fig. 2 and the one in Fig. 4 is the parameter change $r = 0.875$ to $r = 1$. The behavior shown in the figure is of particular interest because of recent reports of hysteresis in the Ca^{2+} activation of isometric force in barnacle fibers (Ridgway et al., 1983; Gordon et al., 1984) as well

as in mammalian skeletal muscle (Gordon et al., 1984; Brandt et al., 1985). Of course, the prediction shown in Fig. 4 is strictly valid only for the biochemical experiments analyzed in this paper since no mechanical aspects have been considered, as would be necessary for a treatment of isometric force (Eisenberg and Hill, 1978; Eisenberg et al., 1980; Hill, 1983). Nonetheless, even though there is some question as to the nature of the hysteresis in mammalian muscle (Brandt et al., 1985), it is worthwhile to discuss the hysteresis predicted here and its relation to that seen in the Ca^{2+} activation of force since this phenomenon represents a break with traditional thinking about muscle contraction and its Ca^{2+} activation.

Since the hysteresis is due primarily to the strong attractive actin-actin interaction found in the analysis here, the arguments for the latter result being an artifact, along with the counterarguments, apply to the prediction of hysteresis as well. Additionally, hysteresis is also predicted by the present work for the binding of myosin to actin; this has not been observed with either two-headed (Greene, 1982) or one-headed (Greene and Eisenberg, 1980; Chalovich et al., 1981) myosin species. On the other hand, the four points presumed to be metastable by the Bethesda school in their analysis of S1 binding (Fig. 2 of Hill et al., 1980; see also Fig. 4 *B* of Hill et al., 1983) are reminiscent of the jump upward in a hysteresis loop. As an additional argument against the prediction of hysteresis being an artifact of the fitting procedures, it can again be noted that the parameters found upon least-squares minimization of the data for the Ca^{2+} binding to regulated actomyosin do not predict hysteresis under the conditions of these experiments, although the initial guesses used to start the least squares routine do.

Two more objections may be raised to the finding of hysteresis, one experimental and the other theoretical. The first is that the shape of the hysteresis loop measured experimentally (Ridgway et al., 1983; Gordon et al., 1984; Brandt et al., 1985) is typical of a pseudo-hysteresis that arises when one does not allow the system to attain true stationary states, i.e., when one simply measures too fast. From the experimental traces, however, it appears that the hysteresis is a true stationary phenomenon, and the authors present additional evidence that this is the case. Another piece of evidence that the hysteresis is experimentally genuine is given by the experiments of Crooks and Cooke (1977) on the Ca^{2+} activation of isometric force in contractile threads; they found that after maximal contraction by exposure to high Ca^{2+}, chelation of the Ca^{2+} by EGTA returned the force only about halfway to baseline. Moreover, hysteresis loops of the typical pseudo-hysteresis form have been seen in calculations for true stationary state (or equilibrium) models (Fig. 4, *bottom* of Shiner and Solaro, 1981). However, the calculated form would correspond to Ca^{2+} binding here, not myosin attachment (and presumably force). Appropriate extensions of the model described here could probably lead to a "pseudo-hysteresis" form for myosin attachment though.

A more fundamental objection to the hysteresis comes from statistical mechanics (Huang, 1983). At thermodynamic equilibrium phase transitions, or hystereses, require the thermodynamic limit, i.e. a large system, with numbers of molecules on the order of, say, moles (10^{23}). A thin filament, presumably the system for the experiments analyzed here, is not a thermodynamic system in this sense, since it has only on the order of hundreds of actin molecules. (This objection would apply less to the hysteresis observed experimentally in whole muscle, since there the system is probably a sarcomere or some other unit much larger than the thin filament.) On the other hand, we are dealing here with hysteresis at nonequilibrium stationary states, and it is not known whether the requirement of the thermodynamic limit from equilibrium statistical mechanics carries over to nonequilibrium stationary states in this case.

A final discrepancy between the present theoretical results and the hysteresis seen experimentally is the following. Fig. 2, *panel C* of Gordon et al. (1984) indicates that the system may have at least three stable stationary states available to it at certain Ca^{2+} concentrations. The theory applied here allows for no more than two stable states, however. Theoretical curves that may have more than two stable states have been calculated in purely theoretical studies (Hill and Stein, 1978), but they require more sophisticated models than the one used here.

Considering all of the above, my own conditional conclusion is that the hysteresis observed in the Ca^{2+} activation of isometric force is authentic, but that the hysteresis predicted for Ca^{2+} binding to regulated actomyosin is probably an artifact, most likely related to the authentic hysteresis, however. In any case, it is a subject which demands more experimental and theoretical study.

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