Activation of Actin-Cardiac Myosin Subfragment 1 MgATPase Rate by Ca^{2+} Shows Cooperativity Intrinsic to the Thin Filament

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ABSTRACT: The magnesium adenosinetriphosphatase (MgATPase) rate of cardiac myosin subfragment 1 (S-1) was studied in the presence of regulated actin in order to investigate the mechanism by which Ca^{2+} cooperatively induces cardiac muscle contraction. The MgATPase rate increased cooperatively with Ca^{2+}, exhibiting a Hill coefficient of 1.8 and 50% activation at pCa 5.75. This cooperative response occurred despite an experimental design excluding several potential sources of cooperativity. First, to exclude spurious cooperativity due to erroneous calculation of pCa at low ionic strength, the affinities of Ca^{2+} and Mg^{2+} for [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) were measured by a novel method using Quin 2. At pH 7.06, 25 °C, and μ = 30 mM, the K_D was 140 nM for CaEGTA and 2.7 mM for MgEGTA. Second, the cooperativity was not produced by actin-myosin S-1 binding; myosin S-1 was bound to only 1 of every 300 actin promoters, and earlier work [Tobacman, L. S., & Adelstein, R. S. (1986) *Biochemistry 25*, 798-802] had shown that cardiac myosin S-1 binds with equal affinity to the thin filament at very low Ca^{2+} and at saturating Ca^{2+} concentrations. Furthermore, the adenosine 5′-triphosphate turnover rate of the myosin S-1 was independent of enzyme concentration at low, intermediate, and saturating Ca^{2+} concentrations. Finally, since cardiac troponin has only one regulatory Ca^{2+}-specific site, cooperative interactions between such sites could not occur. These data suggest that part of the cooperativity conferred by interaction between adjacent troponin-tropomyosin complexes is intrinsic to the thin filament and independent of myosin.

Ca^{2+} induces a cooperative response in both skeletal muscle and cardiac muscle as demonstrated by studies of muscle tension (Hellam & Podolsky, 1969; Julian, 1971; Fabiato & Fabiato, 1978; Brandt et al., 1980; Kerrick et al., 1980; Moss et al., 1986), myofibrillar adenosinetriphosphatase (ATPase)[1] (Solaro & Shiner, 1976; Murray & Weber, 1980), and actomyosin ATPase (Murray & Weber, 1980; Grabarek et al., 1983). Increasing experimental evidence indicates that cooperative Ca^{2+} binding to multiple sites on troponin is not required for this phenomenon. Cooperativity occurs not only in muscles where troponin contains four Ca^{2+} binding sites but also in the heart with three troponin sites (Holroyde et al., 1980) and in crayfish with one troponin site (Wnuk et al., 1984). Furthermore, even on the skeletal muscle thin filament with four Ca^{2+} binding sites, there is little cooperativity in Ca^{2+} binding (Grabarek et al., 1983; Rosenfeld & Taylor, 1985a,b). On the other hand, cooperative actin-myosin binding has become well established and is a widely cited source of muscle protein cooperativity. This cooperative binding of myosin to the thin filament (Bremel et al., 1972) involves interactions between adjacent tropomyosins (Tawada et al., 1975) and has been carefully analyzed experimentally (Greene & Eisenberg, 1980; Trybus & Taylor, 1980; Lehrer & Morris, 1982; Williams & Greene, 1983) and theoretically (Hill et al., 1980, 1983). Studies of muscle fibers variably deficient in troponin C lend in vivo support to this concept of troponin-tropomyosin-mediated cooperativity (Moss et al., 1985; Brandt et al., 1984).

Despite these experimental and theoretical advances, however, there are many gaps in the understanding of cooperative actomyosin ATPase activation by Ca^{2+}. Crucial to any mechanistic interpretation of Ca^{2+}-induced thin filament activation, is whether cooperativity in this activation occurs in the absence of cooperative actin-myosin binding. A number of careful studies of Ca^{2+} binding to troponin (Grabarek et al., 1983; Rosenfeld & Taylor, 1985a,b) do not address the issue of whether the response of the isolated thin filament to Ca^{2+} is cooperative, although Ca^{2+} binding itself is not cooperative under most conditions. Another recent report does address this issue and suggests, on the basis of MgATPase data at low myosin S-1 concentrations, that the thin filament itself responds cooperatively to Ca^{2+} (Walsh et al., 1984). The emphasis of that report, however, concerned a different issue, the comparison between polymerizable and nonpolymerizable tropomyosin.

For three reason, the present report reexamines whether thin filament cooperativity can occur independently of myosin. The first reason is that measured cooperativity can be spuriously affected by unrecognized EGTA impurities or by incorrect metal-EGTA binding constants (Miller & Smith, 1984). Furthermore, with one notable exception (Grynkiewicz et al., 1985), most experiments performed at nonphysiological ionic strength have been analyzed by using unmeasured, arbitrarily extrapolated constants for EGTA. This includes most previous studies of myosin S-1 MgATPase rate vs. $[Ca^{2+}]$. The second consideration is that it requires only very small numbers of either weakly or strongly binding cross-bridges to potentiate binding of these cross-bridges to the thin filament, and different numbers are required in the presence or absence of Ca^{2+} (Greene, 1982; Greene et al., 1986). Such potentiation must be quantitatively excluded before ruling out myosin-induced cooperativity. Finally, this report utilizes cardiac troponin, containing one regulatory Ca^{2+} binding site (Johnson et al.,

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[1] Abbreviations: myosin S-1, myosin subfragment 1; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ATP, adenosine 5′-triphosphate; ATPase, adenosinetriphosphatase.

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1980), instead of skeletal troponin, which contains two. This removes any ambiguities about interactions between such sites inducing cooperativity, so that any observed cooperativity must involve the interaction of one troponin–tropomyosin complex with another.

The results below establish a method for quantifying the degree of Ca^{2+}-induced activation of the thin filament, with a result independent of either actin–myosin or myosin–myosin interactions. As has been necessary for many previous studies using myosin subfragment 1, this work was conducted at relatively low ionic strength; in this case, 30 mM was used. So as to accurately manipulate the Ca^{2+} concentration, a novel method was developed, using Quin 2, to determine the affinity of EGTA for Ca^{2+} and Mg^{2+} under these conditions. This method has general applicability for investigating metal–ligand binding under conditions, such as low ionic strength, not previously studied. With the use of these data, the MgATPase activity of a very low concentration of cardiac myosin subfragment 1, in the presence of actin–troponin–tropomyosin, was studied as a function of the Ca^{2+} concentration. The results indicate a cooperative increase of the MgATPase rate even under these highly controlled conditions. It is suggested that this represents an intrinsically cooperative activation of the thin filament by Ca^{2+}, independent of myosin. The possible mechanism for this cooperativity is discussed, including the significance of its occurrence in this cardiac system with only one Ca^{2+}-specific binding site on troponin.

Materials and Methods

Materials. Bovine cardiac troponin, bovine cardiac tropomyosin, bovine cardiac myosin subfragment 1, and rabbit skeletal muscle actin were purified as previously described (Eisenberg & Kielley, 1974; Tobacman & Adelstein, 1984, 1986). The troponin contains two isoforms of troponin T (Gusev et al., 1983) with the larger form predominating in a 3:1 ratio. Troponin containing either this mixture or only the predominant isoform gave indistinguishable results. Imidazole (grade III) and Quin 2 were purchased from Sigma. EGTA and $SrCl_2$ were obtained from Fluka.

Assays. Fluorescence experiments were performed with an SLM 8000 series spectrofluorometer. For routine assays, slits were at 2 nm, with excitation at 340 nm and emission at 490 nm. ATPase assays utilized the procedure as previously described (Pollard & Korn, 1973) with [γ-^{32}P]ATP produced by NEN, diluted to (2–5) $\times 10^7$ cpm/μmol. The ATPase rates were linear during the first 8–10 min, as determined with five time points.

Ca^{2+} Measurements. A 0.1 M $CaCl_2$ solution (Orion) was used as a standard. EGTA stock solutions were prepared at pH 7.5, and precise concentrations were determined by the turbidimetric method of Miller and Smith (1984). In brief, at pH 9, in the presence of ammonium oxalate, the transition point between clear and turbid occurs when the equivalents of added $CaCl_2$ standard solution exactly equal the equivalents of EGTA. The ratio of volume of EGTA:volume of $CaCl_2$, determined gravimetrically or by buret, is the reciprocal of the ratio of their concentrations. The reproducibility of this method was better than 99%. Stock solutions of EGTA were rechecked or prepared fresh every few weeks, which was necessary to ensure consistent experimental results.

Distilled water was passed through a MilliQ purification system, which includes two ion-exchange cartridges, and, when necessary, through a Chelex column. Remaining Ca^{2+} was checked by measuring the fluorescence intensity of Quin 2, with no addition, with EGTA added, or with $CaCl_2$ added. The free Ca^{2+} concentration ranged between 0.2 and 1.5 μM. This method is based on the observation that Quin 2 has an affinity for Ca^{2+} of about 10^8 at low ionic strength (see below), so essentially all the contaminating Ca^{2+} bound to Quin 2.

The free Ca^{2+} concentration in experimental assays was calculated with an iterative program written to operate with the MLAB program on a DEC10 computer. The following dissociation constants were used (see below): CaEGTA, 140 nM; MgEGTA, 2.8 mM; CaATP, 150 μM; MgATP, 80 μM.

Curve Fitting. In general, algebraic equations were fit directly to the raw fluorescence data, using one adjustable dissociation constant and an adjustable final fluorescence value. This approach avoids overemphasis of errors or artifacts in final fluorescence values and also avoids statistical bias introduced when fitting processed instead of raw data. The MLAB program was used for numerically solving equations and curve fitting, but, of course, any similar program could be used. The fluorescence intensity was fit as the sum of the intensities of the variable concentrations of free Quin 2 (negligible in some experiments), MgQuin 2 (same intensity as free Quin 2 at the wavelengths used), CaQuin 2, and/or SrQuin 2. In the following equations, C, S, M, Q, and E represent total concentrations of Ca^{2+}, Sr^{2+}, Mg^{2+}, Quin 2, and EGTA, respectively. K_{PL} represents the dissociation constant for metal (P) and ligand (L).

The competition of EGTA and Quin 2 for Ca^{2+} is described by

$$[\text{CaQuin 2}] = (C - E - KC - KQ)/(2 - 2K) + [(C - E - KC - KQ)^2 - 4(K - 1)(KCQ)]^{1/2}/(2 - 2K) \quad (1)$$

where $K = K_{CE}/K_{CQ}$, $E > C$, and $Q > C$.

In the displacement of saturating Sr^{2+} from Quin 2 by Ca^{2+}, one can neglect the free Quin 2 concentration and obtain

$$[\text{CaQuin 2}] = \{S + KQ + KC - Q - [(S + KQ + KC - Q)^2 + 4KQC(1 - K)]^{1/2}\}/(2K - 2) \quad (2)$$

where $K = K_{SQ}/K_{CQ}$ and $[\text{SrQuin 2}] = S - [\text{CaQuin 2}]$.

Sr^{2+} binding to unsaturated Quin 2 was studied in the presence of contaminating Ca^{2+} and the presence ($M' \neq 0$) or absence ($M' = 0$) of competing Mg^{2+}. SrQuin 2 is the solution, x, to

$$O = K_{SQ}x + (x - S)[Q - x - F(x) - G(x)] \quad (3)$$

where

$$F(x) = B/(2M' - 2) + [B^2 - 4(1 - M')D]^{1/2}/(2M' - 2)$$

$$G(x) = M'\{Q - x + B/(2 - 2M') + [B^2 - 4(1 - M')D]^{1/2}\}/(2 - 2M')$$

$$M' = M/(K_{MQ} + M), \quad B = (M' - 1)(Q - x + C) - K_{CQ}, \quad D = C(Q - x)(1 - M')$$

Using this numerically found value for $[\text{SrQuin2}] = x$, then the CaQuin 2 concentration, y, is the root of the quadratic

$$O = K_{CQ}y + (y - C)[Q - y - x - M'(Q - x - y)] \quad (4)$$

With both Ca^{2+} and Mg^{2+} competing for Quin 2 and EGTA, and with free $[Mg^{2+}]$ approximated by total $[Mg^{2+}]$, then CaQuin 2 is the root, x, for

$$O = x^2(1 - M') + x[QM' - Q - F(x)M' + CM' + F(x) - C - K_{CQ}] + F(x)QM' - F(x)Q + CQ - CQM' \quad (5)$$

where

$$F(x) = -B/(2 - 2M'') - [B^2 - 4D(1 - M'')]^{1/2}/(2 - 2M'')$$

$$M' = M/(M + K_{MQ}), \quad M'' = M/(M + K_{ME}), \quad D = (M'' - 1)(xE - CE)$$

$$B = (M'' - 1)(C + E - x) - K_{CE}$$

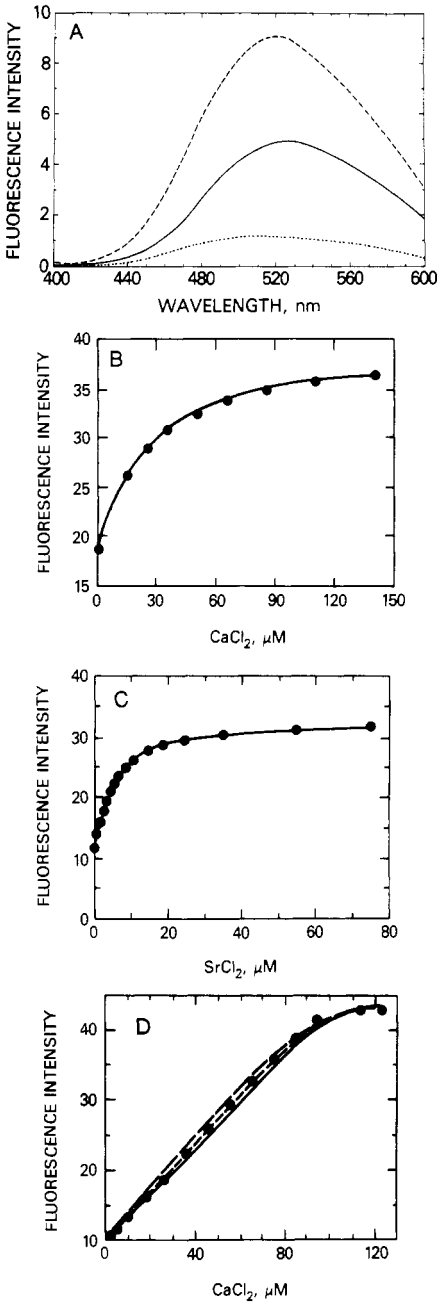

FIGURE 1: Divalent cation binding to Quin 2 and EGTA. (A) Corrected fluorescence emission spectra of 40 μM Quin 2 in the presence of 100 μM $CaCl_2$ (---), 0.88 mM $SrCl_2$ (—), or 1.46 mM EGTA (···), and 20 mM imidazole (pH 7.06) and 20 mM KCl. Excitation was at 339 nm. (B) Displacement of 1.5 mM $SrCl_2$ from 3.6 μM Quin 2 by Ca^{2+} (●) and result predicted from eq 2 with SrQuin 2 K_D:CaQuin 2 K_D = 64:1 (—). Ambient Ca^{2+} concentration was 0.4 μM. Excitation of 339 nM and emission at 490 nm. (C) Binding of Sr^{2+} to 6.3 μM Quin 2 (●) and curve predicted from eq 3 and 4 with 0.35 μM ambient [Ca^{2+}], 0 Mg^{2+}, and SrQuin 2 K_D = 2.6 μM (—). (D) Determination of MgEGTA K_D. Fluorescence data (●); theoretical curves expected from eq 5 with MgEGTA K_D = 4 mM (—), 2.7 mM (---), and 2 mM (--). 97 μM EGTA, 3 mM $MgCl_2$, and 3.6 μM Quin 2 were present. For each panel, the fluorescence intensity is expressed in different, arbitrary units.

RESULTS

Since cardiac myosin S-1 is equally distributed along the thin filament among sites where Ca^{2+} is or is not bound to troponin (Tobacman & Adelstein, 1986), MgATPase activation by increasing Ca^{2+} can be equated to a change, or activation, of the thin filament per se. This would not be the case either if the MgATPase rate were influenced by direct interactions between myosins or if the binding of one myosin to actin altered the behavior of another myosin. The low concentration of myosin S-1 needed to assure the absence of such interactions produces a measurable MgATPase rate only at low ionic strength.

Table I: Binding of Divalent Cations to EGTA and Quin 2

metal	dissociation constants[b]	
	Quin 2	EGTA
Ca^{2+}	40 nM	140 nM
Mg^{2+}	450 μM	2.7 mM
Sr^{2+}	2.56 μM	

[a] Competitive fluorescence titrations were performed as described in the text and dissociation constants obtained by fitting the data to eq 1–5. [b] Conditions: 20 mM imidazole, pH 7.06, 20 mM KCl, 25 °C.

Use of Quin 2 To Measure Metal Binding to EGTA. To accurately control the free Ca^{2+} concentration, the behavior of the Ca^{2+} buffer, EGTA, was measured at 30 mM ionic strength. The affinity of EGTA for Ca^{2+} was determined by fluorometrically measuring the relative affinities of Quin 2 and EGTA for Ca^{2+} and then fluorometrically measuring the affinity of Quin 2 for Ca^{2+}. Quin 2 had a 3.45-fold greater affinity for Ca^{2+} than did EGTA (eq 1; data not shown). This is the same result found at the same pH (7.05) and higher ionic strength, conditions where EGTA's behavior was known and Quin 2's behavior was sought (Tsien et al., 1982). At 30 mM ionic strength, however, the affinity of both EGTA and Quin 2 for Ca^{2+} had to be determined. This was accomplished with the aid of two observations concerning the interaction of Sr^{2+} with Quin 2. First (Figure 1A), SrQuin 2 has a fluorescence intensity conveniently intermediate between that of free Quin 2 and CaQuin 2. Second, CaQuin 2 has an association constant that is 64-fold greater than that of SrQuin 2 (eq 2; Figure 1B). This weaker binding of Sr^{2+} permits direct measurement of its affinity by determining the incomplete binding of Sr^{2+} to Quin 2 at various Sr^{2+} concentrations (Figure 1C). (A similar titration performed with Ca^{2+} shows a linear rise in fluorescence and an abrubt plateau; this gives the number of binding sites, but does not permit quantifying the CaQuin 2 affinity.) The data in Figure 1C were used to measure the absolute values of both these affinities [eq 3 and 4 with $M' = 0$, $G(x) = 0$] by using the relative affinities of Sr^{2+} and Ca^{2+} for Quin 2 (Figure 1B) to correct for contaminating Ca^{2+}. The resultant CaQuin 2 K_D, 40 nM, multiplied by 3.45, gives the desired overall CaEGTA K_D, 140 nM. The validity of these determinations was verified by performing similar titrations at physiological ionic strength. The results (data not shown) agreed with published values for the overall CaEGTA affinity (Martell & Smith, 1974), with H^+ activity and concentration considered (Tsien & Rink, 1980).

The affinity of EGTA for Mg^{2+} was also studied, because high concentrations of Mg^{2+} are required to maximize tropomyosin–actin binding. Furthermore, MgEGTA binding is particularly sensitive to ionic strength (Grynkiewicz et al., 1985). Considerably complicating this measurement was the fact that MgQuin 2 and free Quin 2 have negligibly different fluorescence intensities (Tsien et al., 1982). Determination of the MgQuin 2 K_D was nevertheless relatively straightforward. The experiment shown in Figure 1C was repeated in the presence of 1 and 3 mM $MgCl_2$ (data not shown). More Sr^{2+} was required to saturate the fluorescence change when Mg^{2+} was present to compete for the metal binding site. Fitting the data to eq 3 and 4 established the MgQuin 2 K_D (Table I). To obtain the MgEGTA K_D, however, a double competition experiment was required (Figure 1D), analyzed with eq 5. In this experiment, Ca^{2+} and Mg^{2+} are both competing for EGTA and Quin 2, with a result depending upon

Table II: Effect of Myosin S-1 Concentration on MgATPase Rate[a]

[myosin S-1] (μM)	rate (s^{-1}) at [Ca^{2+}] (M)		
	$<10^{-8}$	10^{-6}	10^{-5}
0.1	0.080	0.457	1.50
0.2	0.081	0.456	1.60
0.3	0.082	0.450	1.55
0.4	0.087	0.437	1.39
0.5	0.092	0.437	1.37
0.6	0.088	0.418	1.48

[a] The MgATPase rate of cardiac myosin S-1 in the presence of regulated actin was measured over a range of enzyme concentrations, at three different Ca^{2+} concentrations. Conditions: 20 mM imidazole, pH 7.06, 3.5 mM $MgCl_2$, 1 mM ATP, 6.5 mM KCl, 0.5 mM EGTA, 24 μM actin, 6.9 μM tropomyosin, 6.9 μM troponin, and $CaCl_2$ ambient, 400 μM, or 500 μM.

all four dissociation constants. Under favorable conditions, the data fit is sufficiently sensitive to the one unknown K_D, that of MgEGTA, for it to be accurately determined. The sensitivity of the fit to this constant is illustrated by three curves corresponding to three K_D values.

Table I summarizes the results of these determinations. All of the dissociation constants are smaller, corresponding to tighter binding, than the usually accepted values at physiological ionic strength. The measured CaEGTA value at 30 mM ionic strength is similar to that obtained if it is approximated by extrapolating the measured value at higher ionic strength, loosely applying the Debye–Hückel theory (Thomas, 1982). MgEGTA binding, however, is more sensitive to ionic strength than would be predicted from this extrapolation. This is consistent with an earlier report that at greater than physiological ionic strength, 250 mM, MgEGTA binding is weakened more than CaEGTA binding (Grynkiewicz et al., 1985). The MgEGTA K_D of 2.8 mM at μ = 30 mM is in the range of Mg^{2+} concentration needed to promote tropomyosin–actin binding. Therefore, at 30 mM ionic strength, Mg^{2+} has an experimentally significant effect on the ability of EGTA to buffer Ca^{2+}. Presumably, this effect is even more pronounced at still lower ionic strength.

MgATPase Rate vs. Myosin S-1. To exclude myosin's well-known ability to cooperatively interact with the thin filament (Bremel & Weber, 1972), the fractional occupation of actin sites by myosin S-1 was kept at a minimum. Although this theoretically could be achieved at physiological ionic strength, where actin–myosin S-1 binding is comparatively weak, this would require use of relatively high myosin S-1 concentrations and consequent problems: (1) high background MgATPase rates in the absence of actin; (2) the possibility that enough denatured myosin S-1 would bind to the thin filament in an ATP-insensitive manner that the interaction of intact myosin S-1 with actin would be altered. On the other hand, at low ionic strength, myosin S-1 binds tightly enough to the thin filament that cooperative binding must be excluded even at low myosin S-1:actin ratios. The experiment shown in Table II shows how this type of thin filament mediated interaction between myosins was excluded. In the presence of 24 μM actin and saturating troponin–tropomyosin, the specific MgATPase activity of the myosin S-1 was virtually independent of S-1 concentration between 0.1 and 0.6 μM. Furthermore, this was true at pCa <8.5 (inhibited), pCa 6 (20% MgATPase activation), or pCa 5 (fully stimulated). Therefore, the degree of MgATPase activation at any Ca^{2+} concentration was independent of cooperative effects of myosin S-1 on its own interaction with the thin filament. In contrast, decreasing the regulated actin concentration from 24 to 6 μM resulted in specific MgATPase rates that increased over the same myosin S-1 concentration range (data not shown).

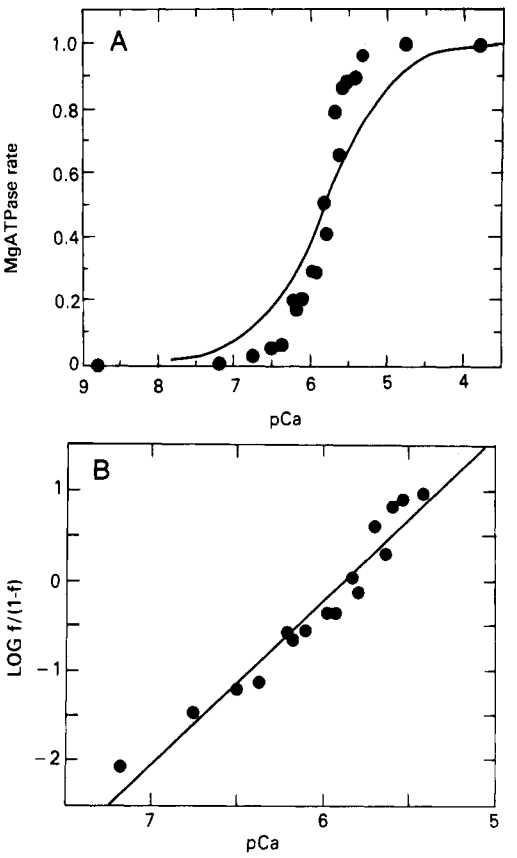

FIGURE 2: Thin filament activated cardiac myosin S-1 MgATPase rate cooperativity increased by Ca^{2+}. (A) MgATPase rates were measured in the presence of 0.3 μM myosin S-1, 24 μM F-actin, 6.9 μM tropomyosin, 6.9 μM troponin, pH 7.06, μ = 30 mM, 25 °C. Basal MgATPase at pCa 9 (4% of the peak value) was subtracted and the data normalized (●). The best fit of the data to the Michaelis–Menten equation is shown (—). (B) Hill plot of the same data shown in part A; n_H = 1.8.

MgATPase Rate vs. Ca^{2+}. By use of the metal–EGTA dissociation constants from Table I and the protein concentrations from Table II, the MgATPase rate of cardiac myosin S-1 was determined as a function of the Ca^{2+} concentration. On the basis of results obtained under similar conditions (Tobacman & Adelstein, 1986), it can be estimated that myosin S-1 was bound to only 1 of every 300 actin promoters. A representative experiment is shown in Figure 2. The midpoint of the curve, at 50% MgATPase activation, always occurred between 1.5 and 2 μM Ca^{2+}, using six different protein preparations. In some experiments, the MgATPase rate fell approximately 10% when the Ca^{2+} concentration was raised from 10 to 100 μM. Despite the careful efforts described above to exclude both artifactual Ca^{2+} concentration errors and cooperativity induced by myosin, the MgATPase rate responded cooperatively to Ca^{2+}. The actual data are clearly distinguishable from the results expected (solid line) if there had been no cooperativity. At Ca^{2+} concentrations less than 0.4 μM, the measured MgATPase rates differed by at least a factor of 3 from those rates expected if there were no cooperativity. The average Hill coefficient for six such experiments was 1.8, with a range between 1.6 and 2. Importantly, the MgATPase rate at intermediate Ca^{2+} concentration is not affected by clustering of myosin S-1 near a subset of troponin molecules which are saturated with Ca^{2+}. Rather, since cardiac myosin S-1 binds equally well to the thin filament at very low Ca^{2+} concentration and at saturating Ca^{2+} concentration (Tobacman & Adelstein, 1986), it should be randomly distributed along the thin filament relative to saturated

or unsaturated troponin.

Discussion

Many examples of cooperative behavior by contractile proteins have been quantitatively analyzed, including actin–myosin binding as detailed above, tropomysin–actin binding (Wegner, 1979), myosin phosphorylation (Sellers et al., 1983), scallop myosin activation (Chantler et al., 1981), and actin polymerization (Tobacman & Korn, 1983). Substantially unexplained, however, is the cooperative response of the regulated actin–myosin MgATPase rate to Ca^{2+}. This is a particularly important consideration for cardiac function, because incomplete Ca^{2+} saturation of cardiac troponin is the rule in vivo (Fabiato, 1981) and also because the heart, in contrast to skeletal muscle, cannot be regulated by variation in the number of motor units contracting with each heart beat. Two recent reports indicate that Ca^{2+} binding to the skeletal muscle thin filament either is not cooperative (Rosenfeld & Taylor, 1985a) or is only cooperative in the presence of saturating myosin S-1 (Grabarek et al., 1983). Other sources of cooperativity must be involved, therefore, in the effect of Ca^{2+}. One such possible source is tropomyosin–tropomyosin interactions, as described in related alternative models (Hill et al., 1980, 1983). These models, put forth to explain detailed studies of actin–myosin binding, also include a myosin-mediated mechanism for a cooperative response to Ca^{2+}. The present study, however, demonstrates cooperativity in MgATPase activation under conditions in which myosin binding is noncooperative; the one form of cooperativity can occur independently of the other. This observation can be incorporated into revised future models of thin filament behavior, particularly if the ability (shown here) to experimentally distinguish thin filament activation by Ca^{2+} per se facilitates further investigation. Specifically, a detailed new model will require comparison between the MgATPase rates reported here and saturation of the Ca^{2+}-specific site on cardiac troponin C.

In summary, there are several reasons to interpret Figure 2 as indicating that the thin filament has an intrinsically cooperative response to Ca^{2+} with a limited number of possible mechanisms. Multiple Ca^{2+}-specific binding sites on troponin, spurious Ca^{2+} determinations, and cooperative myosin S-1 binding to actin were all excluded. Furthermore, the experiment was designed to provide the simplest possible relationship between MgATPase rate and conformational change in the thin filament. Specifically, both the free and the bound myosin S-1 concentrations were unaffected by varying the Ca^{2+} concentration (Tobacman & Adelstein, 1986). Not only was there no cooperativity in myosin S-1 binding but also there was no Ca^{2+}-induced change in myosin S-1 binding. Therefore, the ATPase cooperativity is a true property of the thin filament and is caused by interactions between adjacent units of troponin–tropomyosin–seven actins.

The Hill coefficient of 1.8 for thin filament activation in this report provides a semiquantitative value to compare to previously published data. A very similar Hill coefficient was reported for a similar experiment performed with skeletal muscle proteins (Walsh et al., 1984), suggesting that the additional Ca^{2+} binding site on troponin does not influence the steepness of this type of response. These coefficients are lower than many published results for activation of more intact systems (Brandt et al., 1980). Although both theoretical and experimental considerations could well explain this variability (Levy et al., 1976; Shiner & Solaro, 1982), it is likely that myosin-induced cooperativity, carefully excluded in this report, is another important factor determining muscle behavior.

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Registry No. EGTA·Ca, 52930-59-3; EGTA·Mg, 105900-11-6; Quin 2·Ca, 105900-12-7; Quin 2·Mg, 105900-13-8; Quin 2·Sr, 105900-14-9; ATPase, 9000-83-3; calcium, 7440-70-2.

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Iron(III) Clusters Bound to Horse Spleen Apoferritin: An X-ray Absorption and Mössbauer Spectroscopy Study That Shows That Iron Nuclei Can Form on the Protein†

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ABSTRACT: Ferritin is a complex of a hollow, spherical protein and a hydrous, ferric oxide core of ≤4500 iron atoms inside the apoprotein coat; the apoprotein has multiple (ca. 12) binding sites for monoatomic metal ions, e.g., Fe(II), V(IV), Tb(III), that may be important in the initiation of iron core formation. In an earlier study we observed that the oxidation of Fe(II) vacated some, but not all, of the metal-binding sites, suggesting migration of some Fe during oxidation, possibly to form nucleation clusters; some Fe(III) remained bound to the protein. Preliminary extended X-ray absorbance fine structure (EXAFS) analysis of the same Fe(III)–apoferritin complex showed an environment distinct from ferritin cores, but the data did not allow a test of the Fe cluster hypothesis. In this paper, with improved EXAFS data and with Mössbauer data on the same complex formed with ^{57}Fe, we clearly show that the Fe(III) in the distinctive environment is polynuclear (Fe atoms with Fe–Fe = 3.5 Å and T_B = 7 K). Moreover, the arrangement of atoms is such that Fe(III) atoms appear to have both carboxylate-like ligands, presumably from apoferritin, and oxo bridges to the other iron atoms. Thus the protein provides sites not only for initiation but also for nucleation of the iron core. Sites commodious enough and with sufficient conserved carboxylate ligands to accommodate such a nucleus occur inside the protein coat at the subunit dimer interfaces. Such Fe(III)–apoferritin nucleation complexes can be used to study the properties of the several members of the apoferritin family.

Ferritin maintains iron in a form available to cells for such crucial uses as oxygen transport (the globins), electron transfer and oxygen activation (the cytochromes), nitrogen reduction (nitrogenase), and DNA synthesis (ribonucleotide reductase). The need for ferritin appears to have coincided with the evolution of dioxygen as a byproduct of photosynthesis. Oxygen in the environment prohibits life forms from using the facile metabolism of soluble ferrous and requires the complex uptake, distribution, storage, and recycling of relatively insoluble ferric [the solubility of Fe(III) is approximately 10^{-9} times that of Fe(II) in air at physiological pH (Biederman & Schindler, 1957)].

Thousands (≤4500) of iron atoms may be accommodated in a single ferritin molecule as a polynuclear, hydrous, ferric oxide core inside the hollow apoprotein shell that is formed by the 24 subunits of apoferritin (inner diameter ca. 80 Å in mammalian ferritins [reviewed in Theil (1983, 1987)]). However, in any population of ferritin molecules, a range of core sizes is present. Channels piercing the apoprotein shell appear to provide sites for the entrance and egress of iron. The

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