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Regulation of the Adenosinetriphosphatase Activity of Cross-Linked Actin–Myosin Subfragment 1 by Troponin–Tropomyosin

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ABSTRACT: Chalovich and Eisenberg [Chalovich, J. M., & Eisenberg, E. (1982) *J. Biol. Chem. 257*, 2432–2437] have suggested that at low ionic strength, troponin–tropomyosin regulates the actomyosin ATPase activity by inhibiting a kinetic step in the actomyosin ATPase cycle rather than by blocking the binding of myosin subfragment 1 (S-1) to actin. This leads to the prediction that troponin–tropomyosin should inhibit the ATPase activity of the complex of actin and S-1 (acto·S-1) even when S-1 is cross-linked to actin. We now find that the ATPase activity of cross-linked actin·S-1 prepared under milder conditions than those used by Mornet et al. [Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306] is inhibited 90% by troponin–tropomyosin in the absence of Ca^{2+}. At μ = 18 mM, 25 °C, the ATPase activity of this cross-linked preparation is only about 2-fold greater than the maximal actin-activated ATPase activity of S-1 obtained with regulated actin in the absence of Ca^{2+}. At physiological ionic strength, the ATPase activity of this cross-linked actin·S-1 preparation is inhibited about 95% by troponin–tropomyosin. Since cross-linked S-1 behaves kinetically like S-1 in the presence of infinite actin concentration, it is very unlikely that inhibition of the ATPase activity of cross-linked actin·S-1 is due to blocking of the binding of S-1 to actin. Therefore, these results are in agreement with the suggestion that troponin–tropomyosin regulates primarily by inhibiting a kinetic step in the ATPase cycle.

Vertebrate skeletal muscle contraction is controlled by the Ca^{2+} concentration in muscle; at low levels of Ca^{2+} ($<10^{-6}$ M^{-1}), the muscle is relaxed, and the associated actin-activated ATPase activity of the myosin cross-bridges is inhibited, while increasing the Ca^{2+} concentration reverses this process. This regulation by Ca^{2+} is mediated by the regulatory complex troponin–tropomyosin (Perry, 1979; Weber & Murrary, 1973). Tropomyosin lies along the actin groove of the thin filament, with each tropomyosin molecule binding to seven actin monomers (Ebashi, 1980). Bound to one end of tropomyosin is a troponin molecule, composed of three subunits, one of which binds Ca^{2+}. X-ray diffraction studies have shown that the binding of Ca^{2+} causes a shift in the position of the tropomyosin relative to the actin groove; in the presence of Ca^{2+}, tropomyosin lies in the actin groove, while in the absence of Ca^{2+}, tropomyosin moves away from the groove to a position in which it may be able to interfere with the binding of the myosin cross-bridge on actin (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973). These structural data formed the basis for the steric blocking model which suggests that relaxation is due to tropomyosin physically blocking the binding of the myosin cross-bridge to actin. In a variant of this model, it has also been suggested that troponin–tropomyosin may indirectly block the binding of the cross-bridge to actin by inducing a conformational change in the actin itself (Weber & Murray, 1973).

Recently, the steric blocking model was tested by measuring the binding of myosin subfragment 1 (S-1)[1] to regulated actin

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[1] Abbreviations: S-1, myosin subfragment 1; acto·S-1, complex of actin and S-1; regulated acto·S-1, complex of troponin–tropomyosin and acto·S-1; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAN-BD, 4-[*N*-[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3-benzoxadiazole; HMM, heavy meromyosin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

in the presence of ATP. These in vitro studies showed that, at low ionic strength, troponin–tropomyosin did not significantly affect the binding of S-1 to actin in the presence of ATP under conditions in which it caused marked inhibition of the acto·S-1 ATPase activity (Chalovich et al., 1981; Wagner & Giniger, 1981; Chalovich & Eisenberg, 1982). This led Chalovich and Eisenberg to suggest that troponin–tropomyosin regulates muscle contraction by inhibiting a kinetic step in the actomyosin ATPase cycle. However, several studies still support the steric blocking model of relaxation. Inoue & Tonomura (1982) found that the binding of S-1 to regulated actin in the presence of ATP shows increasing Ca^{2+} sensitivity as the ionic strength is raised. In addition, Wagner and co-workers (Wagner & Giniger, 1981; Wagner & Stone, 1983; Wagner, 1984) have found that the binding of HMM to regulated actin in the presence of ATP shows greater Ca^{2+} sensitivity than the binding of S-1 under identical conditions. Finally, Huxley and co-workers (Huxley et al., 1984, 1985), examining the change in the X-ray pattern after muscle activation, found that the sequential change in the tropomyosin and equatorial reflections is consistent with the steric blocking model.

In the present study, we further investigated the mechanism of muscle regulation by determining the extent to which troponin–tropomyosin inhibits the ATPase activity of S-1 that is chemically cross-linked to actin by EDC (Mornet et al., 1981). Cross-linked actin·S-1 hydrolyzes ATP at a rate comparable to the maximal actin-activated ATPase rate of S-1 (V_{max}) (Mornet et al., 1981; Stein et al., 1985). This indicates that the cross-linked S-1 is behaving kinetically like S-1 in the presence of infinite actin concentration. Just like non-cross-linked S-1 at very high actin concentrations, cross-linked S-1 spends essentially all of its time actually bound to actin in the presence of ATP, instead of only being tethered to actin by the cross-linker. Further evidence in support of cross-linked S-1 being actually bound to actin in the presence of ATP is that increasing ionic strength causes an increase in the ATPase rate of cross-linked actin·S-1 (Stein et al., 1985) even though it markedly weakens the binding of S-1 to actin in the presence of ATP (Greene et al., 1983). Since cross-linked S-1 acts as if it is in an environment of extremely high actin concentration, it might be expected that troponin-tropomyosin would not be able to block its binding to actin. Therefore, the steric blocking model would predict that troponin–tropomyosin should, at best, only poorly inhibit the ATPase activity of cross-linked actin·S-1 and perhaps do so only at high ionic strength where the binding is already weakened. On the other hand, the regulation model proposed by Chalovich & Eisenberg (1982) predicts that troponin–tropomyosin should strongly inhibit the ATPase activity of acto·S-1 even when S-1 is cross-linked to actin since the troponin–tropomyosin acts by blocking a kinetic step in the ATPase cycle. Our results show that, if the cross-linked actin·S-1 is prepared under mild conditions, troponin–tropomyosin causes 90–95% inhibition of its ATPase activity.

Materials and Methods

Rabbit skeletal myosin, S-1, and actin were prepared as described previously (Stein et al., 1979). The molecular weights used for S-1 and actin were 120 000 and 42 000, respectively. Protein concentrations were determined by UV absorption as described previously (Stein et al., 1979). To prepare ^{14}C-labeled S-1, the rabbit myosin was modified with iodo[^{14}C]acetamide according to Greene & Eisenberg (1980) and then digested by chymotrypsin to make S-1. Native tropomyosin was modified with IANBD according to Trybus & Taylor (1980) with the absorbance of IANBD being measured at 480 nm.

To prepare cross-linked actin·S-1, unmodified S-1 was first mixed with iodo[^{14}C]acetamide-labeled S-1 at a 10:1 ratio, with the mixture having a specific activity of $\sim 2 \times 10^{12}$ cpm/mol. The trace amount of radioactive S-1 was used to determine both the ratio of cross-linked S-1 to total actin and the total concentration of the cross-linked preparation (Stein et al., 1985). The method used to cross-link the S-1 to actin, which is described in detail in Stein et al. (1985), is essentially the method of Mornet et al. (1981). This method gives a percentage, on a mole basis, of cross-linked S-1 to total actin of about 20%. We also prepared cross-linked actin·S-1 with a percentage of moles of cross-linked S-1 to moles of total actin of 1% using several different procedures. In the first two procedures, 15 mM EDC was added to activate the actin, just as in the procedure of Mornet et al. The low ratio of cross-linked S-1 to total actin was then achieved either (1) by reacting the S-1 with the EDC-activated actin for only 30 s (instead of 9 min), while keeping the concentrations of S-1 and actin at the same concentrations as in the Mornet et al. preparation, or (2) by reducing the concentration of S-1 that was added to the EDC-activated actin to 0.12 μM (instead of 3 μM), while keeping the time of the reaction at 9 min. Both of these preparations had similar ATPase activities, and the data are compiled together in Table I in the row labeled 1% cross-linked. In the third procedure used to make 1% cross-linked actin·S-1, the cross-linking was done under much milder conditions. We used 0.75 mM EDC to activate the actin, and the cross-linking reaction was at 5 °C, pH 6.25, whereas in the Mornet et al. preparation, 15 mM EDC is used and the reaction is conducted at 20 °C, pH 6.0. We also prepared EDC-treated actin in which the actin was activated with EDC the same as in the 20% cross-linked preparation, but then only buffer, instead of buffer plus S-1, was added to the EDC-activated actin. Regulated cross-linked proteins were routinely prepared by mixing actin with a 150% excess (3 mol/7 mol of actin) of native tropomyosin and dialyzing overnight at 4 °C against 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EGTA, and 4 mM imidazole, pH 7.0. With regulated actin, a 100% excess of troponin–tropomyosin was used.

In the cross-linked preparations, the amount of free S-1 was less than 10% of the amount of cross-linked S-1, as determined by electrophoresis on 10% SDS gels (Laemmli, 1970). In addition, the ATPase activity of the 1% cross-linked preparations was found to double as the ionic strength was raised from 18 to 168 mM, just as with the 20% cross-linked preparation of Mornet et al. This also showed that there was not a significant amount of free S-1 in the 1% cross-linked preparations.

ATPase measurements were performed with the use of an automatic pH stat (Eisenberg & Moos, 1967). Radioactive samples were counted in a Beckman LS-250 scintillation counter. All ATPase rates were increased by 10% to correct for the trace amounts of iodo[^{14}C]acetamide-modified S-1 in the S-1 preparation.

EDC was from Pierce Chemicals, iodo[^{14}C]acetamide was from Amersham/Searle, ATP was from Sigma Chemicals, and IANBD was from Molecular Probes.

Results

To determine how effectively troponin–tropomyosin regulates the ATPase activity of cross-linked actin·S-1, we first determined the extent to which this regulatory complex inhibited the ATPase activity of non-cross-linked acto·S-1. The actin-activated ATPase activity of S-1 in the presence of

Table I: ATPase Activity of Cross-Linked Actin·S-1 at Low Ionic Strength[a]

preparation	rate (s^{-1})		
	unregulated	regulated + Ca^{2+}	regulated − Ca^{2+}
non-cross-linked proteins at V_{max}[b]	23.8	20.0	1.0
20% cross-linked	19.4 ± 1.2 (n = 4)[c]	17.9 ± 0.6 (n = 4)	7.2 ± 0.7 (n = 4)
1% cross-linked	18.5 ± 1.3 (n = 4)	15.3 ± 1.0 (n = 4)	3.9 ± 0.3 (n = 4)
cross-linked under mild conditions	20.0 ± 1.6 (n = 5)	17.0 ± 1.1 (n = 5)	1.9 ± 0.3 (n = 5)

[a] Conditions were 3.5 mM KCl, 3 mM imidazole, 3 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, pH 7.0 at 25 °C, and either 0.5 mM $CaCl_2$ or 1 mM EGTA. [b] Values obtained from the ordinate intercepts of double-reciprocal plots. [c] Data are shown with the standard deviation obtained from n number of cross-linked preparations.

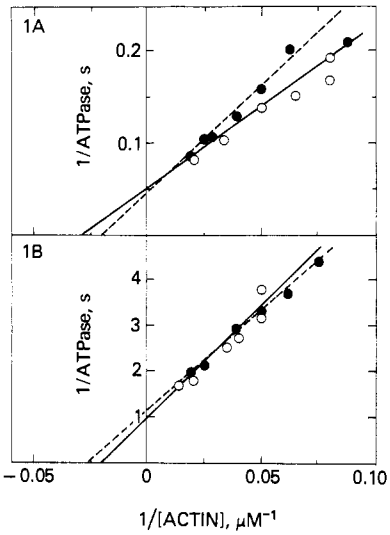

FIGURE 1: Effect of EDC treatment of actin on the regulated actin-activated ATPase activity of S-1 in the presence and absence of Ca^{2+}. ATPase activity was measured under the following conditions: 3.5 mM KCl, 1 mM ATP, 3 mM $MgCl_2$, 1 mM DTT, and 3 mM imidazole, pH 7.0 at 25 °C, in the presence of either 0.5 mM $CaCl_2$ (A) or 1 mM EGTA (B). The actin was either EDC-treated actin (●) or nontreated actin (○). At low regulated actin concentration, S-1 concentration was varied over a 4-fold range to check that the ATPase rate per S-1 did not change. The S-1 concentration was typically 1.5–2.5 μM for assays in the absence of Ca^{2+} and 0.1–0.2 μM for assays in the presence of Ca^{2+}. Linear regression analysis was used to fit the data. The dashed line is the fit obtained for the EDC-treated actin, and the solid line is for the pure actin.

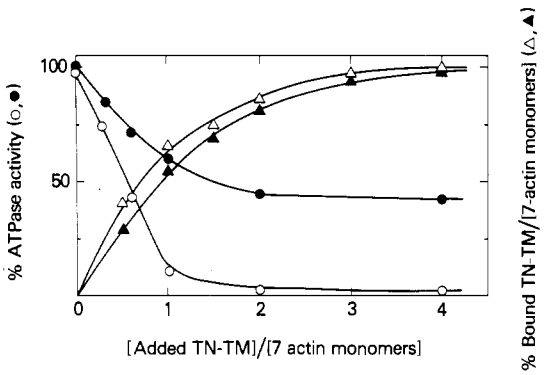

FIGURE 2: Inhibition of actin-activated S-1 ATPase activity and binding of troponin–tropomyosin to actin at varying ratios of troponin–tropomyosin to actin. Conditions are given in Figure 1. In the ATPase measurements, varying ratios of troponin–tropomyosin were added to either cross-linked actin·S-1 (●) or pure actin (○) and then dialyzed overnight. With cross-linked actin·S-1, the ATPase measurements were made by using 0.1–0.2 μM cross-linked S-1. The ATPase rate obtained in the absence of troponin–tropomyosin was 18.4 s^{-1}. With non-cross-linked proteins, the ATPase measurements were obtained with 10 μM actin. In the absence of troponin–tropomyosin, the ATPase activity was 8.6 s^{-1}. In the binding studies, IANBD-modified troponin–tropomyosin was added to either cross-linked actin·S-1 (▲) or pure actin (△). After 30 min at 25 °C, the proteins were centrifuged at 80000g for 1 h at 25 °C, and then the concentration of the IANBD-modified troponin–tropomyosin in the supernatant was determined by measuring its absorbance at 480 nm. The measurements were corrected for the 10% of the troponin–tropomyosin that sedimented upon centrifugation in the absence of actin. TN-TM is troponin–tropomyosin.

troponin–tropomyosin was therefore measured both with and without Ca^{2+} (at μ = 18 mM, 25 °C). The values for V_{max} obtained by using the pH-stat method, which are given in Table I (row 1), are in excellent agreement with those obtained by Chalovich & Eisenberg (1982) using liberation of $[^{32}P]P_i$ from [γ-^{32}P]ATP to measure ATPase activity. With regulated actin, V_{max} was 20 and 1 s^{-1} in the presence and absence of Ca^{2+}, respectively.

To determine the effect of EDC treatment of the actin on the behavior of troponin–tropomyosin, actin was reacted with EDC under conditions identical with those used in the cross-linking procedure of Mornet et al. (1981). As shown in Figure 1, EDC treatment of the actin did not significantly affect either V_{max} or K_{ATPase} values. Similar results were obtained with three different preparations of EDC-treated actin. Therefore, under these conditions, the rate of ATP hydrolysis in the absence of Ca^{2+} is only about 5% of the rate in the presence of Ca^{2+}. However, it should be noted that for reasons we do not yet understand, the low rate in the absence of Ca^{2+} is significantly elevated above the rate of 0.08 s^{-1} obtained for the Mg^{2+}-ATPase activity of S-1 alone.

The regulation of the ATPase activity of cross-linked actin·S-1 by troponin–tropomyosin was then measured under identical conditions using a cross-linked preparation made according to the procedure of Mornet et al. (1981), which had a percentage, on a mole basis, of cross-linked S-1 to total actin of about 20%. In the absence of troponin–tropomyosin, the cross-linked actin·S-1 hydrolyzes ATP at a rate comparable to V_{max} (Table I, row 2). Upon addition of troponin–tropomyosin to this 20% cross-linked preparation, there was only partial regulation of the ATPase activity. In the presence of Ca^{2+}, the rate of the regulated cross-linked preparation was similar to the V_{max} obtained with the non-cross-linked proteins under identical conditions. However, in the absence of Ca^{2+}, the rate of the regulated cross-linked complex was 7 s^{-1}, about 7 times the extrapolated value obtained for non-cross-linked S-1 at infinite regulated actin concentration (Table I). The 20% cross-linked preparation therefore shows rather poor regulation of its ATPase activity by troponin–tropomyosin.

We examined whether this lack of inhibition was due to incomplete saturation of the actin filament with native tropomyosin by measuring the ATPase activity of cross-linked actin·S-1 at varying ratios of troponin–tropomyosin to actin. As shown in Figure 2, the addition of troponin–tropomyosin caused a maximal 60% inhibition of the ATPase activity (closed circles). The extent of this inhibition was not significantly increased by changing the ratio of troponin–tropomyosin to actin from 2:7 to 4:7, which indicated that the actin was saturated with troponin–tropomyosin. A similar pattern of ATPase inhibition as a function of the troponin–tropomyosin

Table II: ATPase Activity of Cross-Linked Actin·S-1 at High Ionic Strength[a]

preparation	rate (s^{-1}) unregulated	regulated + Ca^{2+}	regulated − Ca^{2+}
20% cross-linked	38.2 ± 1.5[b]	45.4 ± 1.6	23.2 ± 2.0
cross-linked under mild conditions	42.2 ± 2.0	39 ± 1.0	2.5 ± 0.5

[a] Conditions were 150 mM KCl, 3 mM imidazole, 3 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, pH 7.0 at 25 °C, and either 0.5 mM $CaCl_2$ or 1 mM EGTA. [b] Data are shown with the standard deviation obtained from three cross-linked preparations.

to actin ratio was obtained with pure actin (open circles).

To confirm these ATPase studies, we also performed direct binding studies of troponin–tropomyosin to cross-linked actin·S-1 using IANBD-modified troponin–tropomyosin. The binding to actin was followed by spectrophotometry, using the absorbance of IANBD to measure the concentration of troponin–tropomyosin, after sedimentation of all the bound troponin–tropomyosin. As shown in Figure 2 (triangles), the binding of troponin–tropomyosin to cross-linked actin·S-1 is very similar to that obtained with pure actin. Upon addition of three troponin–tropomyosin molecules per seven actin monomers, there was one troponin–tropomyosin bound to every seven actin monomers in the cross-linked filament. Therefore, the poor regulation of the 20% cross-linked preparation is not due to partial saturation of the cross-linked actin filament with troponin–tropomyosin.

In an attempt to improve the regulation of the cross-linked complex, we tried many different cross-linking procedures. If we simply decreased the percentage of cross-linked S-1 to total actin to 1% from the 20% found in the Mornet et al. preparation, we improved the regulation by troponin–tropomyosin a little less than 2-fold (row 3, Table I). However, we were only able to obtain a level of regulation with cross-linked proteins comparable to that found with non-cross-linked proteins by using milder conditions of cross-linking. In this method of preparation, the concentration of EDC in the cross-linking reaction was 5% of that used in the procedure of Mornet et al., and the temperature was also reduced to 5 °C. These mild conditions of cross-linking as well as the resulting low percentage of S-1 cross-linked to actin (1% instead of 20%) resulted in troponin–tropomyosin regulating the cross-linked actin·S-1 almost as well as the non-cross-linked S-1. In the presence of troponin–tropomyosin and the absence of Ca^{2+}, the rate of 2 s^{-1} obtained with this cross-linked preparation was only 2-fold greater than the V_{max} of 1 s^{-1} obtained with non-cross-linked S-1, while in the presence of Ca^{2+}, the rate of the regulated cross-linked S-1 was 85% of the V_{max} value obtained with non-cross-linked proteins under identical conditions (Table I). Furthermore, the cross-linked preparations made under mild conditions had the same ATPase activity in the absence of troponin–tropomyosin as the cross-linked preparation of Mornet et al. (1981). Therefore, these results show that troponin–tropomyosin markedly inhibits the ATPase activity of acto·S-1 even when S-1 is cross-linked to actin.

Having obtained a cross-linked preparation which showed good regulation, we examined whether troponin–tropomyosin still inhibited its ATPase activity at physiological ionic strength in the absence of Ca^{2+}. Regulation of the acto·S-1 ATPase activity at physiological ionic strength cannot be measured by using non-cross-linked proteins since without cross-linking the S-1 binds too weakly to actin to give significant actin activation of the S-1 ATPase activity. Table II shows the ATPase rate

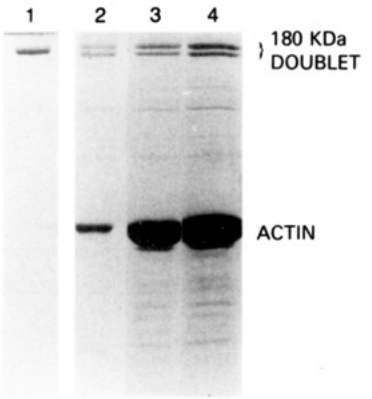

FIGURE 3: SDS gel of cross-linked actin·S-1 preparations. Lane 1, myosin standard; lane 2, 20% Mornet et al. cross-linked preparation loaded with 2 ng of S-1 that is cross-linked to actin; lanes 3 and 4, cross-linked preparations made under mild conditions loaded with 3 and 6 ng of S-1 cross-linked to actin, respectively.

at physiological ionic strength of both the Mornet et al. 20% cross-linked preparation and the cross-linked preparation made under mild conditions. With both cross-linked preparations, the ATPase activity in the absence of troponin–tropomyosin was about double the rate obtained at low ionic strength. In addition, the rate of the regulated cross-linked actin·S-1 in the presence of Ca^{2+} also doubled in both preparations as the ionic strength was increased from 18 to 168 mM. On the other hand, the two preparations responded differently to troponin–tropomyosin in the absence of Ca^{2+}. At μ = 168 mM, the rate of the 20% cross-linked actin·S-1 was 23 s^{-1} in the presence of troponin–tropomyosin and absence of Ca^{2+}, about 3 times the value obtained at 18 mM ionic strength. In contrast, under the same conditions, the rate of the cross-linked preparation made under mild conditions was 2.5 s^{-1}, similar to the rate obtained at low ionic strength. Therefore, at both low and high ionic strength, troponin–tropomyosin causes marked inhibition of the ATPase activity of the cross-linked actin·S-1 preparations made under mild conditions.

Discussion

The extensive inhibition of the ATPase activity of cross-linked actin·S-1 by troponin–tropomyosin does not appear to be consistent with the steric blocking model of muscle regulation. This conclusion depends on two assumptions: first, that the cross-linked S-1 is very tightly bound to actin in the presence of ATP, and second, that troponin–tropomyosin is not able to completely block this very strong binding. Evidence for the first assumption is provided by the finding that cross-linked actin·S-1 hydrolyzes ATP at a rate about equal to V_{max}, which suggests that each cross-linked S-1 molecule spends essentially all of its time bound to actin, just as does non-cross-linked S-1 at very high actin concentration. Even more persuasive, the ATPase activity of the cross-linked preparation doubles as the ionic strength is increased from 18 to 168 mM, while this increase in ionic strength weakens the binding of S-1 to actin by a factor of 100 (Greene et al., 1983). Therefore, it seems very unlikely that the cross-linked S-1 is simply tethered to actin rather than actually being bound to it.

The assumption that troponin–tropomyosin totally blocks the very strong binding of cross-linked S-1 to actin is more difficult to rule out. However, if this were the case, it might be expected that the effect of troponin–tropomyosin would markedly increase as the ionic strength was increased due to the weaker binding of the cross-linked S-1 to actin. However, we observed that increasing ionic strength gave only slightly

better regulation (2-fold) in the ATPase activity of the regulated cross-linked actin·S-1 preparation made under mild conditions, while there was essentially no change in the regulation of the 20% cross-linked preparation. Therefore, it seems unlikely that troponin–tropomyosin is inhibiting the ATPase activity of the cross-linked actin·S-1 by blocking the very tight binding of the cross-linked S-1 to actin.

We found that effective regulation of the cross-linked actin·S-1 only occurred with the cross-linked preparation made under mild conditions and not with the preparation of Mornet et al. (1981). It appears that two factors were responsible for this better regulation. First, lowering the percentage of S-1 cross-linked to actin helped to improve the regulation (Table I). This suggests that high S-1 to actin ratios turn on or potentiate the ATPase activity of cross-linked S-1·actin to some extent just as with non-cross-linked S-1 and regulated actin (Bremel & Weber, 1972; Bremel et al., 1972). However, preliminary experiments measuring the regulation of non-cross-linked S-1 by the 20% cross-linked actin·S-1 show that the thin filament is only being slightly turned on by this cross-linked S-1. This finding is consistent with the apparent lack of cooperativity observed in the binding of S-1 to regulated actin in the presence of ATP (Chalovich et al., 1983); i.e., high S-1 to actin ratios should not shift the position of the tropomyosin and therefore should not turn on the ATPase activity of regulated acto·S-1 to a significant extent.

The second factor responsible for the high level of regulation is the cross-linking procedure itself. This is shown by the fact that the 1% cross-linked preparation made under mild conditions showed much better regulation than the 1% preparation made by the method of Mornet et al., even though both preparations had similar ratios of cross-linked S-1 to total actin. On SDS gels (Figure 3), the cross-linked preparation prepared under mild conditions still had a doublet at 180 kilodaltons on SDS gels, just like the 20% Mornet et al. cross-linked preparation. It may be that the mild conditions of cross-linking reduce the number of amino acids on actin, and perhaps on S-1, that react with EDC, which enables the troponin–tropomyosin to bind in the proper conformation at the acto·S-1 interface. Specifically, there are five acidic residues in the N-terminal fragment of actin which were shown by Sutoh (1982) to be the region of cross-linking to S-1. It is possible that under mild conditions fewer of these residues react with EDC. In addition, there may be other acidic residues on the actin polypeptide chain which react with EDC in the Mornet et al. procedure, but not in the cross-linking procedure under mild conditions. More information about the detailed chemistry of the cross-linking reaction between actin and S-1 will be necessary to understand fully why the mild conditions of cross-linking improve the regulation by troponin–tropomyosin.

Chalovich & Eisenberg (1982) previously showed that, at least up to 50 mM ionic strength, troponin–tropomyosin regulated the acto·S-1 ATPase cycle by blocking a kinetic step. However, they could not work at physiological ionic strength because of the weak binding of S-1 to actin. Our results obtained with cross-linked actin·S-1 at physiological ionic strength suggest that here too troponin–tropomyosin regulates by blocking a kinetic step in the acto·S-1 ATPase cycle. These results do not rule out that troponin–tropomyosin also has an effect on the binding of the myosin cross-bridge to actin in vivo. Nevertheless, our results strongly suggest that inhibition of a kinetic step in the actomyosin ATPase cycle, perhaps P_i release, plays a major role in relaxation at physiological ionic strength.

It is often suggested that the P_i release step is associated with the rotation of the cross-bridge from the 90° to the 45° conformation. Since troponin–tropomyosin effectively regulates cross-linked S-1, our data raise the possibility that such a conformational change may be able to occur even when S-1 is cross-linked to actin. Direct evidence in support of this idea was recently presented in the electron microscopy study of Craig et al. (1985). The structure of cross-linked actin·S-1 was found to be very disordered in the presence of ATP, with individual S-1 molecules attaching to actin at variable angles centering on 90°, while in the absence of ATP, cross-linked actin·S-1 showed the typical arrowhead appearance of the rigor conformation. Biochemical studies are currently under way using the effect of troponin–tropomyosin on the cross-linked actin·S-1 to obtain further evidence that the cross-linked complex can undergo an oscillation between two conformations.

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Isoleucyl-tRNA Synthetase from Bakers' Yeast: Multistep Proofreading in Discrimination between Isoleucine and Valine with Modulated Accuracy, a Scheme for Molecular Recognition by Energy Dissipation

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ABSTRACT: For discrimination between isoleucine and valine by isoleucyl-tRNA synthetase from yeast, a multistep sequence is established. The initial discrimination of the substrates is followed by a pretransfer and a posttransfer hydrolytic proofreading process. The overall discrimination factor D was determined from k_{cat} and K_m values observed in aminoacylation of $tRNA^{Ile}$-C-C-A with isoleucine and valine. From aminoacylation of the modified tRNA species $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′NH_2), the initial discrimination factor I (valid for the reversible substrate binding) and the proofreading factor P_1 (valid for the aminoacyl adenylate formation) could be determined. Factor I was computed from ATP consumption and D_1, the overall discrimination factor for this partial reaction which can be obtained from kinetic constants, and P_1 was calculated from AMP formation rates. Proofreading factor P_2 (valid for aminoacyl transfer reaction) was determined from AMP formation rates observed in aminoacylation of $tRNA^{Ile}$-C-C-A and $tRNA^{Ile}$-C-C-3′dA. From the initial discrimination factor I and the AMP formation rates, discrimination factor D_{AMP} in aminoacylation of $tRNA^{Ile}$-C-C-A can be calculated. These values deviate by a factor Π from factor D obtained by kinetics which may be due to the fact that for acylation of $tRNA^{Ile}$-C-C-A an initial discrimination factor $I' = I\Pi$ is valid. The observed overall discrimination varies up to a factor of 16 according to conditions. Under optimal conditions, 38 000 correct aminoacyl-tRNAs are produced per 1 error while the energy of 5.5 ATPs is dissipated. With the determined energetic and molecular flows for the various steps of the enzymatic reaction, a coherent picture of this new type of "far away from equilibrium enzyme" emerges.

The frequency by which valine may substitute for isoleucine in vivo has been determined in direct measurements to be about 1:3000 (Loftfield, 1963; Loftfield & Vanderjagt, 1972). To achieve this high specificity of protein biosynthesis, all enzymes involved in the translational process should exhibit the same specificity since the step with the lowest accuracy determines the overall fidelity.

A first conjecture about the possible accuracy which may be achieved by an enzyme in discrimination between isoleucine and valine was made by Pauling (1958). Starting from the assumption that the smaller amino acid valine should also fit into the binding site of isoleucine, Pauling calculated the van der Waals attraction energy with which the additional methylene group of isoleucine might interact with the enzyme surface. According to the London theory of electronic dispersion forces, a value of about 0.9 kcal/mol (3.77 kJ/mol) was found which corresponds to a discrimination factor of 4.3 in favor of isoleucine. This value was confirmed by measurements of the interaction of methyl-substituted antigens with antibodies (Pauling & Pressman, 1945).

In a similar calculation, Hopfield & Yamane (1979) estimated the optimal difference in binding energy between isoleucine and valine to be about 2.5 kcal/mol (10.46 kJ/mol, corresponding to a discrimination of 1:60) on the basis of the assumption that the critical methyl group of isoleucine might be surrounded by 6–9 methyl equiv.

To obtain directly the discrimination factor caused by different binding energies, the kinetic constants k_{cat} and K_m were measured for both substrates in the ATP/PP_i pyrophosphate exchange reaction which represents the reverse of the first reaction step of the aminoacylation reaction:

$$E^{Ile} + aa + ATP \rightleftharpoons E^{Ile}\cdot aa\text{-}AMP + PP_i$$

$$E^{Ile}\cdot aa\text{-}AMP + tRNA^{Ile} \rightleftharpoons E^{Ile} + aa\text{-}tRNA^{Ile} + AMP$$

For the discrimination factor $D_{PP_i} = (k_{cat}/K_m)_{Ile}(k_{cat}/K_m)_{Val}^{-1}$, values of about 200 were calculated due to a difference of about 3 kcal/mol (12.6 kJ/mol) in the free binding enthalpies of the two substrates (Fersht, 1977a, b, 1981; Fersht et al., 1980). In a different approach, $tRNA^{Ile}$-C-C-3′dA and $tRNA^{Ile}$-C-C-A(3′NH_2) were aminoacylated with isoleucine and valine, and from k_{cat} and K_m values, similar discrimination factors were obtained (Cramer et al., 1979; Freist & Cramer, 1983). These discrimination factors were considered to be due to the binding energy of the additional methylene group of isoleucine.

Because the discrimination factors mentioned above are too low to explain the high in vivo accuracy, the enzyme must act

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