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Does ATP cause relaxation by binding to actin or its regulatory proteins?

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SUMMARY

Although it has been shown that troponin and tropomyosin are not able to cause relaxation at low ATP concentration suggesting that they require ATP for their activity, neither regulatory protein nor actin bind ATP.

For relaxation, vertebrate actomyosin systems require ATP in relatively high concentrations; at low concentrations of ATP contraction persists even in the absence of calcium[1-3]. In order to account for this ATP requirement several investigators have postulated the existence of special binding sites for ATP responsible for relaxation[2-4]. If such binding sites were to exist one would expect to find them on one of the proteins of the regulated actin filament since it has been shown that relaxation results from the turning off of the active site of actin, abolishing interaction with nucleotide-containing myosin[5]. We, therefore, measured ATP binding to calcium-regulated, *i.e.* troponin and tropomyosin containing actin filaments. We now document our finding on which we briefly reported at a symposium in 1970 [5].

We prepared calcium-regulated thin filaments by polymerizing purified actin with the tropomyosin–troponin complex as it is contained in Ebashi's preparation of "native" tropomyosin[6]. After assembly, these filaments were isolated from the mixture by centrifugation. The saturation of each filament preparation with troponin and tropomyosin was evaluated from the calcium dependence of ATPase activity after the addition of purified myosin. Over the range of ATP concentrations used for binding measurements calcium activated the rate of ATP hydrolysis 10-fold (Table I). With relaxation thus 90% complete we would expect 90% saturation of the putative ATP-binding sites. Depending on which

TABLE I

Mg-ATP AND Ca^{2+} BINDING AND COFACTOR ACTIVITY OF REGULATED THIN FILAMENTS

Preparation	*Mg-ATP (μM)*	*ATP hydrolysis* (μmoles/min per mg myosin)*		*ATP binding** (ATP/actin monomer)*	*Calcium binding*** (Ca^{2+}/actin monomer)*
		$-Ca^{2+}$	*$+Ca^{2+}$*		
1	25	0.07	0.72	0.011	
	38	0.07	0.65	0.011	0.31
	44	0.11	0.70	0.019	
2	43	0.04	0.57	0.001	
	68	0.06	0.60	0.000	0.52
	100	0.06	0.56	0.000	
3	29			0.012	
	58	Not		0.010	0.48
	87	Measured		0.008	
	116			0.010	

* After mixing regulated filaments with myosin in a weight ratio of 1/3 ATPase activity was determined either in the presence of 2 mM EGTA ($-Ca^{2+}$) or 0.1 mM $CaCl_2$ ($+Ca^{2+}$) and with ATP concentrations as indicated. Other conditions of the assay were 2.0 mM $MgCl_2$; 2.0 mM creatine phosphate; 1.25 mg/ml creatine kinase; 10 mM imidazole pH 7.0; 0.07 ionic strength; 0.3 mg/ml actomyosin. Creatine liberation was measured after 1 min incubation at 25 °C.

** After mixing 2–4 mg/ml protein of regulated filaments with ^{14}C-labeled Mg-ATP of known specific activity in concentrations as indicated, in the presence of 1.0 mM EGTA, 3.0 mM creatine phosphate + 1.0 mg/ml kinase, 1.0 mM $MgCl_2$, 10 mM imidazole, pH 7.0, the filaments were separated by 90 min centrifugation at 100 000 × *g*. Binding was calculated from the radioactivity in the pellet after correcting for free solute either by weighing or by using [^3H]glucose as a marker for free solute. The actin content of the filaments was calculated from the amount of unexchangeable ADP, assuming 1 mole ADP per mole actin monomer (Tsuboi, 1968).

*** After mixing 1.5–1.8 mg/ml regulated filaments with 17–19 μM ^{45}Ca (= added + exchangeable calcium contained in the preparation, the latter was removed by EGTA from a control sample and measured by atomic absorption, using internal standards) in the presence of 3.0–7.0 mM imidazole, pH 7.0, 0.08 M KCl and 1.0 mM $MgCl_2$, the filaments were separated and the bound calcium calculated as for the measurements of ATP binding.

protein of the regulated filament requires ATP for its function the number of expected binding sites may equal maximally the number of actin monomers and minimally the number of troponin or tropomyosin molecules. Since polymerized actin does not bind calcium exchangeably (pure actin was found to take up maximally 0.015 mole calcium per mole actin) the troponin content can be estimated from the amount of exchangeable calcium bound to the filaments at saturating calcium concentrations (Table I). This number divided by 4 represents the maximal number of troponin molecules since each troponin has 4 calcium-binding sites[7]. The number of tropomyosin molecules cannot be smaller than that of troponin[8].

The data in Table I show that ATP binding to calcium-regulated filaments was negligible. Even if all of the ATP taken up by preparation 1, the preparation which bound most, were associated with troponin or tropomyosin, only 0.25 of the molecules would

have contained ATP compared to an 86% inhibition of ATPase activity on removal of calcium. However, it seems more likely that this ATP was not bound to either of the regulatory proteins but instead had been incorporated into the polymerized actin since it could not be washed out after resuspending the filaments in 100 mM KCl. After polymerization actin-bound ADP becomes unexchangeable[9], except at sites where the polymer breaks due to mechanical agitation. At these sites ADP exchanges for ATP which is converted back to ADP on closing of the polymer break[10,11]. When we were careful to avoid mechanical agitation as much as possible the amount of ATP bound became very small.

It appears therefore that ATP cannot act directly on any component of a regulated actin filament. This conclusion agrees well with the observation that calcium regulation does not depend uniquely on the ATP concentration. The ATP concentration required for complete calcium regulation is quite variable and depends on the ratio of myosin active sites to actin[7]. We have concluded that ATP functions by reducing the concentration of nucleotide-free myosin which is capable of combining with actin in the absence (or presence) of calcium. Formation of such complexes between actin and myosin turns on the filaments for contraction through protein–protein interactions[7] releasing them from control by troponin and calcium. The only ATP-binding site that is required for this role in relaxation is the well known hydrolytic site of myosin.

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