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Cryoenzymic Studies on Actomyosin ATPase. Evidence That the Degree of Saturation of Actin with Myosin Subfragment 1 Affects the Kinetics of the Binding of ATP†

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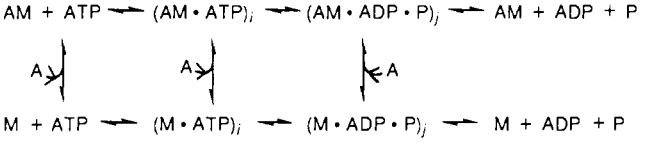
Received July 28, 1989; Revised Manuscript Received October 6, 1989

ABSTRACT: The initial steps of actomyosin subfragment 1 (acto-S1) ATPase (dissociation and binding of ATP) were studied at −15 °C with 40% ethylene glycol as antifreeze. The dissociation kinetics were followed by light scattering in a stopped-flow apparatus, and the binding of ATP was followed by the ATP chase method in a rapid-flow quench apparatus. The data from the chase experiments were fitted to E + ATP $\rightleftharpoons$ (K_1) E·ATP $\rightarrow$ (k_2) E*ATP, where E is acto-S1 or S1. The kinetics of the binding of ATP to acto-S1 were sensitive to the degree of saturation of the actin with S1. There was a sharp transition with actin nearly saturated with S1: when the S1 to actin ratio was low, the kinetics were fast ($K_1 > 300$ μM, $k_2 > 40$ s^{-1}); when it was high, they were slow ($K_1 = 14$ μM, $k_2 = 2$ s^{-1}). With S1 alone $K_1 = 12$ μM and $k_2 = 0.07$ s^{-1}. With acto heavy meromyosin (acto-HMM) the binding kinetics were the same as with saturated acto-S1, regardless of the HMM to actin ratio. The dissociation kinetics were independent of the S1 to actin ratio. Saturation kinetics were obtained with $K_d = 460$ μM and $k_d = 75$ s^{-1}. The data for the saturated acto-S1 could be fitted to a reaction scheme, but for lack of structural information the abrupt dependence of the ATP binding kinetics upon the S1 to actin ratio is difficult to explain. It is tentatively proposed that the transition is due to a cooperative phenomenon involving head–head interaction. It is suggested that in unsaturated acto-S1 the heads do not interact, even when attached to adjacent actin monomers, but that as further S1 binds and the saturation nears completion there is an abrupt structural change of the filament which is propagated along it. This is then transmitted to the heads which now interact. Therefore, it is proposed that when the heads interact, the ATP binding kinetics are slow (apparently this is always the case with acto-HMM) but when they do not, the kinetics are fast. The kinetic transition is not due to the presence of ethylene glycol: it was also obtained in an aqueous buffer with N^6-ethenoadenosine 5′-triphosphate. The results are discussed with reference to the structural information available in the literature.

Muscle contraction—as do the several processes involved in cell motility—depends on the cyclic interaction of actin and myosin, the energy for which is supplied by the hydrolysis of ATP[1] by the myosin heads. It is thought that movement is controlled by actin interacting in different ways with the different intermediates on the myosin ATPase reaction pathway. Therefore, to understand muscle contraction, one must obtain detailed information on the kinetics of the several processes involved in actomyosin ATPase.

Scheme I



It is very difficult to study directly the kinetic processes of organized systems. The use of caged compounds is an elegant way of attacking this problem [e.g., Goldman et al. (1984)

†This work was supported by a grant to C.T. from the European Economic Community (Stimulating action), Contract 85200162UKOHPUJU1.
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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ε-ATP, 1, N^6-ethenoadenosine 5′-triphosphate; P_i, inorganic orthophosphate; S1, myosin subfragment 1; HMM, heavy meromyosin; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

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and Hibberd and Trentham (1986)]. Nevertheless, solution studies remain the main source of precise kinetic information on actomyosin ATPase.

When ATP interacts with actomyosin (rigor complex) in solution, there are two initial events: the actomyosin complex dissociates, and the ATP is tightly bound before being finally hydrolyzed. Conventionally, actomyosin ATPase is described by the two parallel pathways in Scheme I, one for actomyosin, the other for myosin [for reviews, see Trentham et al. (1976) and Taylor (1979)], where M is myosin (or one of its proteolytic subfragments) and A actin. The number of intermediates i and j vary according to the citation [e.g., Lymn and Taylor (1972), Eisenberg and Greene (1980), Geeves et al (1984), and Millar and Geeves (1988)]. The scheme was derived from solution studies, but the results of recent work show that, at least in part, it applies to muscle fibers too (Hibberd & Trentham, 1986).

When all of the published work is taken account of, Scheme I involves several tens of intermediates, and with the methods available it is very difficult to obtain all of the kinetic constants involved under a single set of experimental conditions.

There are a number of experimental parameters that can modulate the actomyosin pathway. Thus, variation in ionic strength, pH, solvent composition, or temperature or in the concentration of ATP, actin, or myosin can influence the relative amounts of the different intermediates. Under certain conditions the experimental data lead to a very simplified version of Scheme I, under others to more elaborated ones. This may explain controversies as to the number and dispositions of the different intermediates of actomyosin ATPase. However, perturbing a system by changing the experimental conditions can be a useful means of obtaining mechanistic information. By this means one can separate temporally phenomena that appear to occur simultaneously under "normal" conditions.

Here we consider the beginning of the actomyosin pathway, namely, the dissociation process and the steps preceding hydrolysis of the ATP. Even these seemingly elementary steps have been shown to be composite [e.g., Sleep and Taylor (1976), Millar and Geeves (1983), and Biosca et al. (1984)]. To increase the information content of our experiments, we exploit temperature and solvent perturbation.

The data were obtained by the use of two methods. In the first, the dissociation of acto-S1 was followed by light scattering in a stopped-flow apparatus [e.g., White and Taylor (1976)]. In the second, the binding of ATP was followed by the ATP chase method (Trentham et al., 1976).

Unexpectedly, we find that with acto-S1 the kinetics of the binding of ATP is sensitive to the degree of saturation of the actin with S1. There was a sharp transition with actin that was nearly saturated with S1: when the S1 to actin ratio was below 1 (unsaturated acto-S1), the kinetics were fast, but when the ratio was 1 (saturated acto-S1), they were slow. The dissociation process did not vary with the degree of saturation of actin with S1. With acto-HMM there did not appear to be a transition—the binding kinetics were the same as with saturated S1, regardless of the degree of saturation of the actin with HMM. Finally we confirm our previous results (Biosca et al., 1984), namely, that the conformation of the S1 freshly released from acto-S1 is different from that of S1 alone.

Materials and Methods

Proteins and Reagents. References to the preparation of myosin from rabbit skeletal muscle and its proteolytic fragments S1 and HMM are in Tesi et al. (1989). Actin was prepared following the method of Spudich and Watt (1971). [γ-^{32}P]ATP was from Amersham International. ϵ-ATP (Sigma) was purified as in Tesi et al. (1988).

Experimental Conditions. Except where otherwise stated, all experiments with ATP were carried out at −15 °C with 40% ethylene glycol as antifreeze in 5 mM Tris, 5 mM KCl, 2 mM magnesium acetate, and 1 mM DTT, adjusted to pH 8 with acetic acid. The experiments with ϵ-ATP were carried out at 4 °C in the same buffer but without ethylene glycol. Air bubbles were removed from acto-S1 solutions by centrifugation at low speed on a desk centrifuge for 1 min.

Cold ATP Chase Method. This is based upon the observation that on the reaction pathway of S1 ATPase there is a quasi-irreversible isomerization of an S1·ATP complex before the hydrolysis step (Goody et al., 1977). This is illustrated in Scheme II, where E represents S1, HMM, or their complexes with actin. The ATP-induced dissociation of acto-S1 or acto-HMM was not considered here.

Scheme II

$$E + ATP \rightleftharpoons E{\cdot}ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*{\cdot}ATP \xrightarrow{k_{cat}} E + ADP + P$$

The feasibility of the ATP chase method rests upon the relationship $k_{-2} < k_{cat} \ll k_2$ (Trentham et al., 1976). Therefore, by its use one measures "tightly" bound ATP.

The procedure is as follows: Reaction mixtures containing acto-S1 or acto-HMM and [γ-^{32}P]ATP were aged in a rapid-flow quench apparatus (Barman & Travers, 1985) and quenched in a large excess of unlabeled ATP. The mixture was incubated for 3 min at 20 °C and finally quenched in 20% trichloroacetic acid containing 1 mM KH_2PO_4 and the $^{32}P_i$ determined (Reimann & Umfleet, 1978).

The $^{32}P_i$ determined can be expressed as

$$[^{32}P_i]/[\text{active site}] = [1 - \exp(-kt)] + k_{cat}t$$

where

$$k = k_2[ATP]/([ATP] + K_1) \quad (1)$$

This equation (Barman et al., 1983) predicts that after a rapid exponential rise in $^{32}P_i$ (giving k), there is a straight line (steady state) which on extrapolation to zero time gives an amplitude equal to the active-site concentration. k_{cat} is obtained by dividing the steady-state rate by the active-site concentration. For maximum accuracy the ratio of [[γ-^{32}P]ATP] to [myosin head] in the reaction mixture was kept in the range 4–10.

Stopped-Flow Experiments. There were two types of experiment: turbidimetric (dissociation of acto-S1 by ATP or ϵ-ATP) and fluorescence (binding of ϵ-ATP to S1 or acto-S1). They were carried out in a Union Ginken RA401 stopped-flow apparatus with a modified mixing device adapted to cryoenzymic work (Hooper et al., 1983). Further details of experiments with ϵ-ATP are in Tesi et al. (1988).

In dissociation experiments, the decomposition of acto-S1 by ATP or ϵ-ATP is followed by a decrease in turbidity at 360 nm, and the data were interpreted according to Scheme III. The data from the experiments with ϵ-ATP were interpreted according to Tesi et al. (1988).

Scheme III

$$AM + ATP \underset{}{\overset{K_d}{\rightleftharpoons}} AM{\cdot}ATP \xrightarrow{k_d} A + MATP$$

Treatment of Data. Data fitting and simulations were carried out on an Apple IIe computer (Biosca et al., 1984). The data from the stopped-flow experiments were stored in a Sord Mark III computer and transferred to the Apple for treatment.

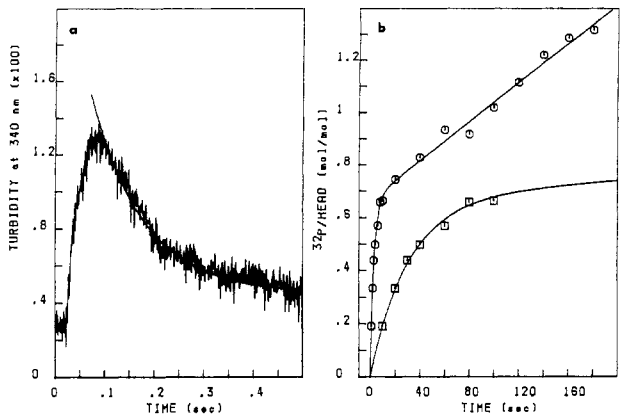


FIGURE 1: Time courses for acto-S1 dissociation and binding of ATP at −15 °C. (a) Dissociation. The reaction mixture was 5 μM S1, 12 μM actin, and 100 μM ATP. The solid line was fitted to 9.5 s^{-1}. (b) Binding of ATP as followed by the ATP chase method. The reaction mixture was 1 μM S1, 20 μM actin, and 3 μM ATP. The solid line was obtained by fitting the data to eq 1, giving k = 0.33 s^{-1}, [active site] = 0.66 mol/mol of S1 and $k_{cat} = 5.7 \times 10^{-3}$ s^{-1}. For points (○) time scale is as indicated; for (□) it is divided by 10.

RESULTS

Typical dissociation and ATP chase experiments with acto-S1 at −15 °C are illustrated in Figure 1. Under the experimental conditions used, the binding of S1 to actin is tight ($K_d \leq 0.05$ μM; Tesi, Travers, and Barman, unpublished results). Thus, in all experiments with [S1] < [actin], all the S1 was bound.

In the dissociation experiment, the process appeared to be complete and the kinetics monophasic. In the ATP chase experiment there was a rapid transient burst phase of amplitude of 0.66 mol of ATP/mol of acto-S1. In the absence of actin the same S1 gave a burst of amplitude of 0.69 (curve not shown). These amplitudes are almost identical, and we assume that as at 15 °C (Biosca et al., 1984, 1985) the ATP chase method gives direct information on the tight binding of ATP to acto-S1.

We note that actin activates considerably the kinetics of the tight binding of ATP to S1. Thus, at 3 μM ATP, k = 0.025 s^{-1} for S1 and 0.32 s^{-1} for acto-S1.

Concentration Dependence of the Dissociation of Acto-S1 for ATP at −15 °C. The dependence curve is illustrated in Figure 2a. The relationship is hyperbolic which shows that the dissociation of acto-S1 by ATP occurs in at least two steps. This confirms the conclusion of Millar and Geeves (1983). The dissociation of several non skeletal muscle actomyosins by ATP also takes place in two steps [references cited in Millar and Geeves (1983)]. Under our experimental conditions, K_d = 460 μM and k_d = 75 s^{-1}. Millar and Geeves (1983) report 8 mM and 121 s^{-1}, respectively, at −5 °C and in a buffer of relatively high ionic strength.

The degree of saturation of the acto-S1 complex with S1 had no detectable effect upon the dissociation kinetics.

Effect of the Saturation of Actin with S1 upon the ATP Dependence of the Tight Binding Kinetics. The dependence of the kinetics of the tight binding of ATP to acto-S1 with actin unsaturated in S1 is shown in Figure 2b. In these experiments the ratio of S1 to actin was in the range 0.5–0.05, and at a given ATP concentration the kinetics were independent of this ratio within the range used. The binding dependence followed closely the dissociation dependence up to at least 160 μM ATP (here k = 20 s^{-1}). Whereas saturation in the dissociation kinetics was obtained, this was not possible in the ATP chase. Experiments at ATP > 160 μM require solutions of high concentrations of acto-S1, and these are too viscous for efficient mixing in our equipment. We have already discussed the problem of mixing viscous solutions in our flow quench apparatus (Barman & Travers, 1985). With it at −15 °C it was possible to attain 20 μM acto-S1 without mixing problems in either mixer.

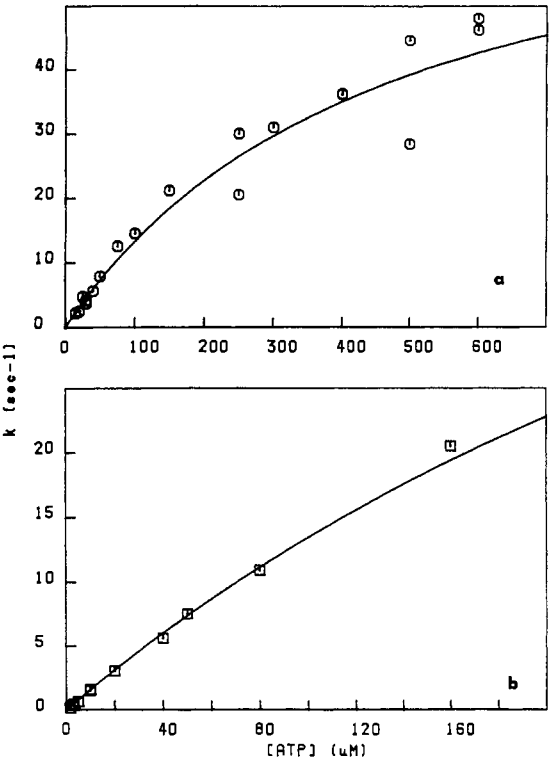


FIGURE 2: Dependencies of the rates of dissociation and binding of ATP to unsaturated acto-S1 upon the ATP concentration at −15 °C. (a) Dissociation. The continuous line was fitted to the data by using Scheme III, giving K_d = 460 (±100) μM and k_d = 75 (±10) s^{-1}. (b) Binding. The continuous line represents the ATP dependence for the dissociation, i.e., the curve in (a).

Table I: Kinetic Constants for the Interaction of ATP with S1 and Acto-S1 at −15 °C[a]

process	material	constant: K_1 or K_d (μM)	constant: k_2 or k_d (s^{-1})
binding[b]	S1	12 (±3)	0.072 (±0.01)
	saturated acto-S1	14.3 (±3.6)	2.15 (±0.3)
	unsaturated acto-S1	>300	>40
dissociation[c]	saturated or unsaturated acto-S1	460 (±100)	75 (±10)

[a] The buffer was 5 mM Tris, 5 mM KCl, and 2 mM magnesium acetate, pH 8, with 40% ethylene glycol as antifreeze. [b] From ATP chase experiments (Figure 3) according to Scheme II (K_1 and k_2). [c] From stopped-flow (turbidimetric) experiments (Figure 2a) according to Scheme III (K_d and k_d).

The ATP dependence of the binding of ATP to acto-S1 saturated in S1 is illustrated in Figure 3. In comparison with the unsaturated complex at high ATP concentrations the binding kinetics were slower and the dependence was hyperbolic. This difference is a significant result, and it confirms our preliminary experiments at 15 °C (Biosca et al., 1984). In particular with the saturated complex the binding kinetics are slower than the dissociation kinetics. Also included in Figure 3 is the dependence upon the ATP concentration of the binding of ATP to S1. To emphasize the slowness of the kinetics, the data are given on the same scales as those for the acto-S1 complexes. The dependence curves were interpreted according to eq 1, and the constants obtained are summarized in Table I.

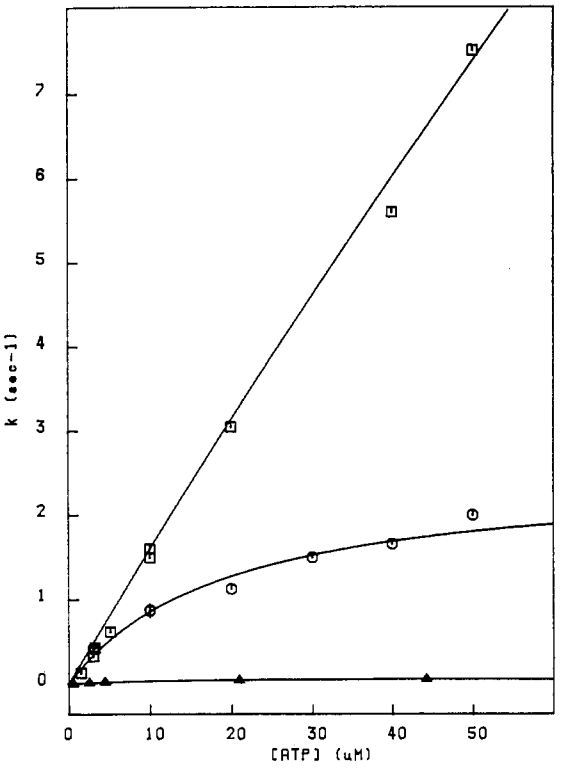


FIGURE 3: Dependencies of the rates of binding of ATP to S1 and acto-S1 upon the ATP concentration at −15 °C. For S1 and saturated acto-S1 the data were fitted to Scheme II: (△) S1, K_1 = 12 (±3) μM and k_2 = 0.072 (±0.01) s^{-1}; (○) saturated acto-S1, K_1 = 14.3 (±3,6) μM and k_2 = 2.15 (±0.3) s^{-1}. For unsaturated acto-S1 (□) the continuous line represents the ATP dependence of the acto-S1 dissociation (Figure 2).

Table II: Effect of the Degree of Saturation of Actin with Myosin Heads upon the Binding of ATP to Acto-HMM and Acto-S1 at −15 °C[a]

	k (s^{-1}) with actin			
	saturated in		unsaturated in	
[ATP] (μM)	S1	HMM	S1	HMM
30	1.5	1.3	4.2	1.9
50	1.7	2.2	7	1.9

[a] From ATP chase experiments in the buffer given in Table I. The values for k with acto-S1 are from Figure 3. With HMM, at 30 μM ATP, HMM and actin equal 7 μM (saturated) or HMM equals 7 μM and actin equals 21 μM (unsaturated). At 50 μM ATP, HMM and actin equal 10 μM (saturated) or HMM equals 15 μM and actin equals 30 μM (unsaturated).

An important feature of the ATP binding kinetics to acto-S1 is that they depend critically on the S1 to actin ratio in a very narrow range. This is illustrated in Figure 4. There is a sharp downward jump in k as the saturation increases from 0.8 to 1 mol of S1/mol of actin. We note that in the region just before saturation k is very sensitive to the S1 to actin ratio. These experiments were carried out at 50 μM ATP, and the binding kinetics appeared to be monophasic in all of them.

ATP Chase Experiments with Acto-HMM. The effect of the degree of saturation of acto-HMM with HMM upon the kinetics of the binding of ATP was studied at 30 and 50 μM ATP, and the results obtained are summarized in Table II. Also included are the data for acto-S1 under identical conditions.

It is noteworthy that with acto-HMM the degree of saturation of actin by the myosin heads had little effect upon the ATP binding kinetics: at both concentrations of ATP the kinetics were slow and similar to those with acto-S1 saturated in S1. The kinetics of the binding of ATP to HMM alone are identical with those with S1 (Tesi et al., (1989).

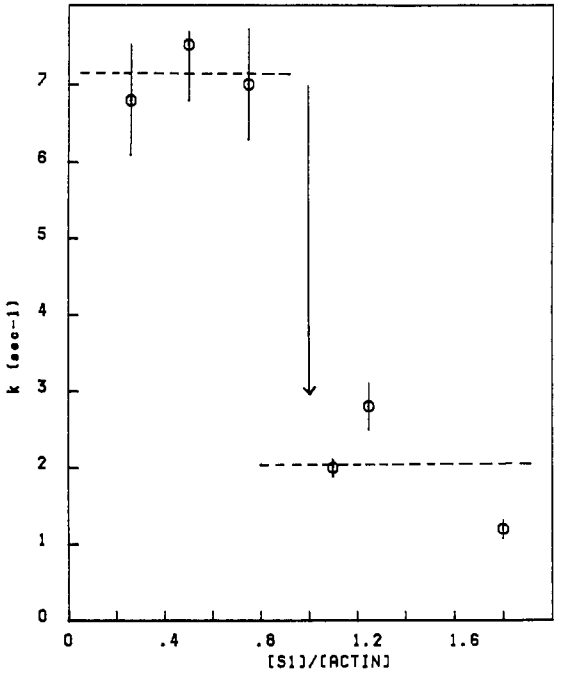


FIGURE 4: Dependence of the ATP binding kinetics upon the S1 to actin ratio in acto-S1 at −15 °C. The data are fom ATP chase experiments at 50 μM ATP with acto-S1 of different degrees of saturation in S1.

Dissociation and Binding Experiments with ϵ-ATP in Water. The finding that the kinetics of the tight binding of ATP to acto-S1 depend on the degree of saturation of the actin with S1 is based on experiments carried out in ethylene glycol. This means of perturbing the system slowed down the otherwise very rapid binding process, which allowed us to observe the saturation effect. It was important to confirm the phenomenon in water. Another way of perturbing the acto-S1 system and thus gaining kinetic information is to replace ATP by the analogue ϵ-ATP (Tesi et al., 1988). The effects of the concentration of ϵ-ATP on the kinetics of the dissociation and its binding to acto-S1 at different saturations in S1 are illustrated in Figure 5. These experiments, which were carried out in water at 4 °C, were difficult, and the differences observed were less than those with ATP at −15 °C. Nevertheless, it appears that the degree of saturation of the acto-S1 with S1 affects the ϵ-ATP binding but not the dissociation kinetics. Thus, with saturated acto-S1 the binding kinetics are slower than with the unsaturated complex. Further, in the concentration range of ϵ-ATP used, the dissociation and binding kinetics to unsaturated acto-S1 were undistinguishable. As with ATP, experiments with ϵ-ATP and nearly saturated acto-S1 were difficult. The kinetics were sometimes slow, sometimes fast, and great care had to be taken with the accuracy of the S1 and actin concentration.

Discussion

Two Types of Acto-S1 Complex. We show that the kinetics of the binding of ATP to acto-S1 depend critically upon the degree of saturation with S1 (Figure 4). This is a surprising result, and we must address ourselves to its causes. In particular, its abruptness and its absence with acto-HMM need to be explained.

Consider the results obtained with acto-S1. When the S1 to actin ratio approached 1, there was an abrupt change in the ATP binding kinetics. This transition may occur under

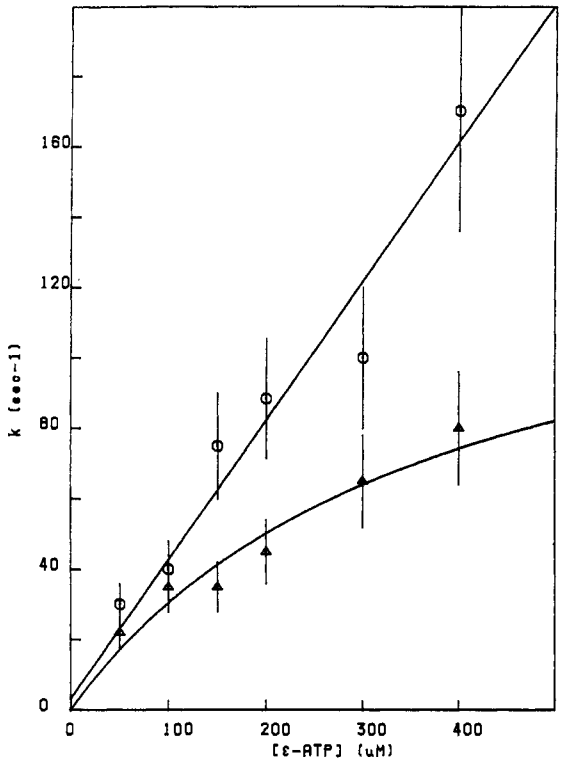


FIGURE 5: Dependencies of the rates of dissociation and binding of ϵ-ATP to acto-S1 in water at 4 °C. (○) Binding with unsaturated acto-S1 (reaction mixture: 5 μM S1 plus 10 μM actin). The continuous line represents the ϵ-ATP dependence of the acto-S1 dissociation (experimental points not shown). (△) Binding with saturated acto-S1 (reaction mixture: 5 μM S1 plus 4 μM actin). The continuous line is the best fit to the experimental points and Scheme III, giving K_1 = 360 (±180) μM and k_2 = 140 (±40) s^{-1}. The buffer used was 5 mM Tris, 5 mM KCl, and 2 mM magnesium acetate, in water, pH 8. The binding data were obtained from fluorescence transients with excitation at 290 nm and emission at 340 nm.

different conditions: in ethylene glycol at −15 °C (here) or at 15 °C (Biosca et al., 1984) and also in water at 4 °C (here) with ϵ-ATP. These results suggest that the kinetics of the binding of ATP to acto-S1 are modulated by a cooperative structural change in the acto-S1 complex as it becomes saturated in S1.

There are several reports describing changes in the properties of acto-S1 as a function of different ranges in the S1 to actin ratio. These studies were carried out by different techniques [e.g. Fujime and Ishiwata (1971), Lascalzo et al. (1975), Ando and Scales (1985), and Ando (1987)]. In particular, Ando (1987), who used rather special conditions, observed a sharp transition in light scattering as the saturation of actin with S1 approached completion. The author termed this "hyperopalescence", which he attributed to the formation of a certain type of bundle. It is uncertain as to whether this is a consequence of a structural transition in acto-S1 or vice versa. Now, this structural transition may occur under different experimental conditions, but it may lead to bundles only under very special conditions such as those used by Ando. Therefore, it is possible that Ando's bundles and our kinetic transition are different manifestation of the same phenomenon: both observations occur abruptly and near saturation of actin in S1.

What is the nature of the actin-induced change in S1 that leads to the change in its kinetic properties? The answer may be in the data from the experiments with actin complexed with the double-headed HMM. HMM appears to bind more strongly to actin than S1, which suggests that both the heads bind (Greene, 1981; Miyata et al., 1989). The two heads may bind to different actin filaments, giving rise to bundles (Trinick & Offer, 1979). On the other hand, using a zero-length cross-linker, Onishi et al. (1989) conclude that the heads of the duplex HMM can be cross-linked when attached to actin but not when they are on their own. This suggests that with HMM the myosin heads bind in pairs to adjacent monomers on the same filament. It seems likely that both intra- and interfilament interactions occur, their relative importance depending upon the particular experimental conditions used.

We return to S1. From their electron microscope studies Vibert and Craig (1982) suggest that S1 molecules attached to adjacent actin monomers are almost in contact. Further, Labbe et al. (1982) could cross-link chemically pairs of S1 molecules in the presence of actin. Therefore, it appears that with saturated acto-S1 there is interaction between the heads, which would mimic the situation with acto-HMM.

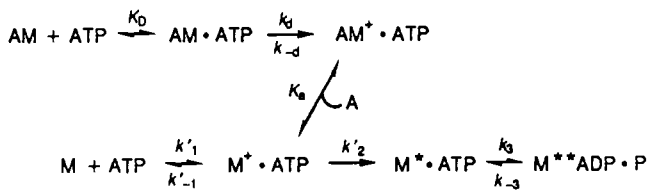
With unsaturated acto-S1 the situation is more difficult. At physiological ionic strengths S1 binds cooperatively to actin, i.e., at low S1 to actin ratios, regions of the actin filament are saturated with S1, whereas others are bare. But at low ionic strengths (as here) the heads appear to bind randomly (Curmi et al., 1988). Thus, it seems unlikely that at low ionic strengths there is interaction between the heads in acto-S1 with a low S1 to actin ratio. It follows that an explanation for the dependence of the ATP binding kinetics upon the degree of saturation of the acto-S1 with S1 is that when the heads are in contact (as in saturated acto-S1 or acto-HMM), the ATP binding kinetics are slow, but when they are not in contact (as in unsaturated S1), they are fast. The dissociation kinetics are identical and fast for the two types of complex (Table I).

From the effect of ionic strength upon the S1-actin interaction (Curmi, 1988), a way of providing further evidence for head–head interaction suggested itself. Thus, if at high ionic strengths and low ratios S1 binds cooperatively to actin, then head interactions should occur and the ATP binding should be slow. There is a suggestion from the work of Millar and Geeves (1988) that this may be so. At high ionic strengths they found by fluorescence stopped-flow techniques that the kinetics of the binding of ATP-γS to unsaturated acto-S1 were slower than the dissociation. At low ionic strengths with ATP these processes are identical with the unsaturated acto-S1 (Figure 3). Unfortunately, we were unable to confirm the ionic strength effects with ATP using the cold chase method: at high ionic strengths K_1 (Scheme II) increases, making such experiments impossible with the equipment available.

It is difficult to propose a cause for the abruptness of the acto-S1 transition: only structural studies will provide a solution. A tentative explanation implies that the heads do not interact even when close to each other, which is the case with ratios greater than 0.5 where certain of the heads are on adjacent actin monomers. When further S1 is added and saturation nears completion, there is a sudden structural change that leads to head–head interaction involving *all* the heads. This would be in accord with Ando (1989), who proposes that under certain conditions when S1 binds to the actin filament, there is a structural change in the filament which is propagated along it and transmitted to all the heads.

Mechanisms for Acto-S1. With the saturated acto-S1 complex we determined the ATP dependencies in the dissociation and tight binding kinetics at −15 °C, and there was a saturation in both. The important point here is that the kinetics of the two processes are different, with the dissociation being faster than the binding kinetics, which in turn are faster than the kinetics of the binding of ATP to S1 alone (Figure 3). This confirms our results at 15 °C (Biosca et al., 1984), but here we amplify these differences. Further, in the earlier

Scheme IV



work we did not obtain saturation in the dissociation process.

As at 15 °C, at −15 °C transient lag phases were not discerned in the ATP chase experiments, even at concentrations of ATP at which the kinetics of dissociation are just faster than those of the ATP chase. Although a lag phase is often difficult to detect, with our data both here and at 15 °C we feel that it is unlikely that we have missed it. Therefore, our results cannot be fitted to a simple scheme involving successive intermediates with dissociation proceding binding.

We have already discussed two schemes to explain the absence of lags in the ATP binding kinetics: one involves two sites for ATP and the other only one (Biosca et al., 1984). Here we consider these schemes with reference to our present results.

In the two-site scheme acto-S1 has one site for dissociation and another for tight binding and hydrolysis. There are certain papers supporting such a scheme; these we have already discussed. As at 15 °C our present data can be fitted to this scheme with the constant given in Table I. However, for want of structural evidence we prefer a more conventional one-site situation.

In the second scheme we introduced a new conformation of S1, the M^+ state. Such a state was considered by Sleep and Taylor (1976), but later it was thought to be unnecessary [e.g., Taylor (1979)]. This was added to explain the difference between the dissociation and tight binding kinetics (see above). Thus, when acto-S1 is dissociated by ATP, the M^+ state is released rapidly and it binds tightly ATP relatively slowly but more rapidly than S1 alone. As the chemical step ($k_3 + k_{-3}$) appears not to change significantly with actin, the M^+ conformation then isomerizes to M^*, a conformation on the pathway of S1 alone. Millar and Geeves (1988) came to the same conclusions. Scheme IV summarizes these findings. The dissociation of M^+·ATP was introduced to explain the lack of transient lag phases in the ATP chase experiments. This is not a fundamental feature of the scheme; in any event it is difficult to obtain unique values for k'_1 and k'_{-1}. Since the kinetics of the dissociation process are independent of the actin concentration (at least within the actin range used), $K_a \gg$ [actin] and k_{-d} could not be obtained.

Although Scheme IV can be used to fit our data, the observed dissociation constant for the ATP chase (e.g., Figure 3) cannot be interpreted in a simple way. Thus, with this scheme, at low ATP concentrations k is low because the dissociation process is rate limiting.

We now turn to the data obtained with the unsaturated acto-S1 complex. Here the salient features are, first, in the concentration range of ATP used, the dissociation and ATP chase kinetics are similar, if not identical. Second, at high ATP concentrations, unsaturated acto-S1 binds ATP at least 500 times faster than does S1 alone. This is a remarkable activation. These results can be explained by the dissociation and binding occurring in one step. But the data can also be fitted to Scheme IV. In this event the dissociation is rate limiting, and the head liberated is different from the M^+ state. We note that with cross-linked acto-S1 too the chase kinetics are as rapid as the unsaturated acto-S1 kinetics (Biosca et al., 1985). This makes sense, as cross-linked acto-S1 is unsaturated in S1 (Mornet et al., 1981).

It is noteworthy that in most of the published work on the kinetics of acto-S1 the unsaturated complex was used. For example, when Rosenfeld and Taylor (1984) studied the dissociation and tight binding processes with ϵ-ATP, identical kinetics were obtained—as expected, as they used a ratio of S1 to actin of 0.6.

We are at present unable to decide which of the acto-S1 complexes—if indeed either—is important in muscle contraction. This remains a subject for further study.

In conclusion, our cryoenzymic studies allowed for the detection of two types of acto-S1 complex that differ in their degree of saturation in S1. Further, they permitted us to obtain certain of the features of a plausible reaction pathway for acto-S1. It was solvent and temperature perturbation that allowed us to amplify the two phenomena, but it seems unlikely that it was this device that caused them. Thus, with ϵ-ATP under "normal" aqueous conditions there was a difference in the ATP binding kinetics to unsaturated and saturated acto-S1 (Figure 5). Further, Millar and Geeves (1988) confirmed the M^+ conformation in water.

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Transfer of Preformed Terminal C5b-9 Complement Complexes into the Outer Membrane of Viable Gram-Negative Bacteria: Effect on Viability and Integrity

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Received June 29, 1989; Revised Manuscript Received September 29, 1989

ABSTRACT: An efficient fusion system between Gram-negative bacteria and liposomes incorporating detergent-extracted C5b-9 complexes has been developed that allows delivery of preformed terminal complexes to the cell envelope (Tomlinson et al., 1989b). Fusion of *Salmonella minnesota* Re595 and *Escherichia coli* 17 with C5b-9-incorporated liposomes resulted in the transfer of 1900 C5b-9 complexes to each target bacterial cell. No loss in viability of bacteria was observed following fusion, even though the deposotion of 900 complexes onto the envelope following exposure to lysozyme-free serum effected a greater than 99% loss of viability. Increased sensitivity to antibiotics normally excluded from the cell by an integral outer membrane (OM), as well as the ability of the chromogenic substrate PADAC to gain access to periplasmically located β-lactamase, indicated that transferred C5b-9 complexes functioned as water-filled channels through the OM. A similar conclusion was drawn from measurements demonstrating the uptake by cells of the lipophilic cation tetraphenylphosphonium (bromide), a result further indicating that the membrane potential across the cytoplasmic membrane was maintained following C5b-9 transfer to the OM. Examination of *S. minnesota* Re595 by electron microscopy revealed no obvious difference between cells exposed to lethal concentrations of lysozyme-free serum and cells following fusion with C5b-9-incorporated liposomes. These data suggest either that there are critical sites in the OM to which liposome-delivered C5b-9 complexes are unable to gain access or that bacterial cell death is related to events occurring during polymerization of C9 on the cell surface.

The cell envelope of Gram-negative bacteria consists of three essential layers: the outer membrane that contains LPS[1] in the outer leaflet and forms the major permeability barrier of the cell, the peptidoglycan layer responsible for cell rigidity, and the cytoplasmic membrane, which possesses functions relating to the transport of nutrients, to oxidative phosphorylation, and to the synthesis of cell envelope and periplasmic macromolecules. Activation of the complement system by susceptible Gram-negative bacteria results in the generation and stable deposition into the OM of terminal C5b-9 complement complexes, events strongly correlated to complement-mediated bacterial cell death (Joiner et al., 1982a,b; Kroll et al., 1984; Taylor & Kroll, 1985). However, there is considerable evidence to suggest that bacterial killing is dependent upon perturbation of the CM [reviewed by Taylor (1983)], and the question as to how complement exerts its lethal effect on the CM from its initial location on the OM remains unanswered. C9 appears to be directly responsible for the cytotoxic effect of complement on Gram-negative bacteria, and the presence of multiple copies of C9 in individual C5b-9 complexes on the bacterial surface has been shown to

[‡] Addenbrooke's Hospital.
[§] Supported by an SERC CASE Post-Graduate Studentship.
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[1] Abbreviations: C5b-9, terminal membrane attack complex of complement; BAS, bentonite-absorbed serum; NHS, normal human serum; NHS-C9, NHS depleted of C9; BC1-8, bacteria bearing complement proteins C1 to C8; CAPT buffer, 130 mM NaCl, 10 mM sodium acetate, 10 mM Na_2HPO_4, 5 mM Tris-HCl, pH 7.0; CFU, colony-forming unit; CM, cytoplasmic or inner membrane; OM, outer membrane; NaDOC, sodium deoxycholate; GVB^{2+}, veronal-buffered saline containing 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$; $GGVB^{2+}$, GVB^{2+} containing 2 mg/mL glucose; LPS, lipopolysaccharide; PADAC, 7-(2-thienylacetamido)-3-[[2-[[4-(*N*,*N*-dimethylamino)phenyl]azo]pyridinio]-methyl]-3-cephem-4-carboxylic acid; TPP^+, tetraphenylphosphonium (bromide); Complement proteins are named in accordance with recommendations in *Bull. W. H. O.* (1968).

0006-2960/90/0429-1852$02.50/0