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Transient Kinetics of Adenosine 5′-Triphosphate Hydrolysis by Covalently Cross-Linked Actomyosin Complex in Water and 40% Ethylene Glycol by the Rapid Flow Quench Method†

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ABSTRACT: The initial steps of the ATPase of covalently cross-linked actomyosin subfragment 1 (acto-SF-1) were studied by the rapid flow quench method, and the results obtained were compared with those with reversible (i.e., non-cross-linked) acto-SF-1 and SF-1 under identical conditions. Cross-linked acto-SF-1 plus [γ-^{32}P]ATP reaction mixture milliseconds old was quenched either in a large excess of unlabeled ATP (ATP chase) or in acid (P_i burst). The conditions were pH 8 and 15 °C at 5 mM or 0.15 M KCl and with or without 40% ethylene glycol. In 40% ethylene glycol (5 mM KCl), as with SF-1 and reversible acto-SF-1, the ATP chase was used to titrate active sites and to study the kinetics of ATP binding. Unlike those with SF-1 or reversible acto-SF-1, saturation kinetics were not obtained. The second-order rate constant for ATP binding was 3.1×10^6 M^{-1} s^{-1} for cross-linked acto-SF-1, 1.8×10^6 M^{-1} s^{-1} for reversible acto-SF-1, and 2×10^6 M^{-1} s^{-1} for SF-1. In P_i burst experiments, a transient phase could not be discerned. Because of a high k_{cat}, cross-linked acto-SF-1 was difficult to study in aqueous solution, but at 5 mM KCl, the ATP chase and P_i burst curves were similar to those obtained in 40% ethylene glycol. At 0.15 M KCl the ATP chase curve was difficult to interpret (small amplitude), and there was a small P_i burst. The data obtained in 40% ethylene glycol (5 mM KCl) were adjusted to an abbreviated form of the Bagshaw–Trentham scheme for myosin:

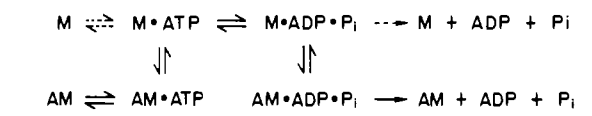
$$AM + ATP \overset{K_1}{\leftrightarrow} AM{\cdot}ATP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AM^*{\cdot}ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} AM^{**}{\cdot}ADP{\cdot}P_i \xrightarrow{k_4} AM + ADP + P_i$$

The best fit was obtained with $K_1k_2 = 3.1 \times 10^6$ M^{-1} s^{-1} ($K_1 < 1.06 \times 10^4$ M^{-1}, $k_2 > 200$ s^{-1}), $k_3 = k_{-3} = 4$ s^{-1}, and $k_4 = 20$ s^{-1}. With SF-1, $K_1 = 1.25 \times 10^5$ M^{-1}, $k_2 = 16$ s^{-1}, $k_3 = k_{-3} = 8$ s^{-1}, and $k_4 = 0.12$ s^{-1} under the same conditions. Thus, it appears that whereas the kinetics of the ATP binding and the release of products for cross-linked acto-SF-1 and SF-1 are very different, the kinetics of their chemical steps are similar.

Muscle contraction 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 the contraction process is modulated by the various intermediates on the ATPase pathway. Therefore, for a full understanding of muscle contraction this pathway must be elucidated.

A reaction pathway for actomyosin ATPase was first proposed by Lymn & Taylor (1971). A key feature of this is that actin dissociates before hydrolysis occurs (Scheme I).

Scheme I[a]



[a] Actomyosin ATPase (dissociative pathway) is indicated by solid arrows. Myosin ATPase is given by the top line. M is myosin and A is actin.

More recent work on the two pathways has lead to an elaboration of the Lymn & Taylor scheme [for reviews, see Trentham et al. (1976), Taylor (1979), Adelstein & Eisenberg (1980), and Sleep & Smith (1981)]. In particular, it is thought that a key step on the myosin pathway is a confor-

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMPPNP, 5′-adenylyl imidodiphosphate; P_i, inorganic orthophosphate; SF-1, myosin subfragment 1; Tris, tris(hydroxymethyl)aminomethane.

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mational change leading to the tight binding of ATP (Bagshaw & Trentham, 1973; Mannhertz et al., 1974). This important feature has allowed the study of ATP binding by the ATP chase technique (Bagshaw & Trentham, 1973; Chock & Eisenberg, 1979; Taylor, 1979), but because of the rapidity of the process, it has been difficult to determine the kinetic constants involved.

A way of reducing this rapidity is to include 40% ethylene glycol in the reaction medium (Barman et al., 1983). This solvent was first used with myosin by Brahms & Kay (1962), and by its use we confirmed the earlier findings of Bagshaw et al. (1974), namely, that ATP binds to myosin subfragment 1 (SF-1) in a two-step process, and we determined the kinetic constants involved under different experimental conditions (Barman et al., 1983; Biosca et al., 1983).

We extended these studies to actomyosin subfragment 1 (acto-SF-1; Biosca et al., 1984a). From our results we concluded, first, that the steps by which ATP dissociates and binds tightly to acto-SF-1 are kinetically distinct. Second, it appears that the conformation of SF-1 freshly released from acto-SF-1 is different from that of SF-1 alone.

A problem with experiments with acto-SF-1 is that the complex is reversible, and it is difficult experimentally to fully saturate the SF-1 with actin in the steady state. A way of overcoming this problem is to cross-link the two proteins chemically [Mornet et al., 1981a; also see Sutoh (1982, 1983), Greene (1984), Heaphy & Tregear (1984), and Arata (1984)]. Since covalently cross-linked acto-SF-1 has an ATPase activity close to that of SF-1 at infinite actin, it appears to be a good model for reversible acto-SF-1. Therefore, we thought it fit to extend our pre-steady-state kinetic studies on SF-1 and reversible acto-SF-1 to covalently cross-linked acto-SF-1.

There are several precise questions concerning the mechanism of cross-linked acto-SF-1 ATPase, in particular the initial steps. Does ATP bind as tightly to this complex as it does to SF-1 or to reversible acto-SF-1? In the covalent complex, the ATPase presumably proceeds via a nondissociative pathway (as has been proposed for the reversible complex at high actin concentration; Stein et al., 1979)—how does this affect the chemical step? What is the predominant intermediate in the steady state?

Certain preliminary studies have appeared on the pre-steady-state kinetics of cross-linked acto-SF-1 (Webb & Trentham, 1982; Stein et al., 1983). Here we extend our rapid flow quench studies on SF-1 and reversible acto-SF-1 to cross-linked acto-SF-1. We show, first, that ATP binds tightly to cross-linked acto-SF-1, whose active sites can thus be titrated, and, second, that the kinetics of the chemical step are similar to those obtained with SF-1 alone.

Materials and Methods

Materials. Myosin SF-1 was prepared from rabbit muscle following Weeds & Taylor (1977) and purified on Sephacryl S-200 (Mornet et al., 1981b). Actin was prepared from rabbit muscle following Spudich & Watt (1971).

Cross-linked acto-SF-1 complex was prepared following Sutoh (1983) or Mornet et al. (1981a) and analyzed by gel electrophoresis following Mornet et al. (1981b). The patterns of the two materials thus prepared were similar, and differences could not be detected from the pre-steady-state experiments described below.

The amount of SF-1 in cross-linked acto-SF-1 was measured by difference, i.e., by subtracting the free SF-1 eliminated by the washing procedures from the SF-1 initially present (Mornet et al., 1981a). Typically, 30–40% of the SF-1 initially present was cross-linked. The amount of unbound SF-1 in the preparations of the cross-linked SF-1 was less than 8%.

Quenched-Flow Experiments. The quenched-flow apparatuses used have already been described (Barman et al., 1980). These devices had been designed to mix solutions of different viscosities. With them, efficient mixing was obtained on mixing 68% glycerol (20 cP) with water (Barman & Travers, 1985), a situation that simulates the mixing of cross-linked acto-SF-1 with ATP.

ATP chase and P_i burst experiments were carried out as described previously for reversible acto-SF-1 (Biosca et al., 1984a) with certain modifications. In ATP chase experiments, reaction mixtures containing [γ-^{32}P]ATP were quenched in a 1000-fold molar excess of unlabeled ATP, incubated for exactly 20 s at 15 °C, and quenched in 5.4% trichloroacetic acid, and the [^{32}P]P_i was determined (Reimann & Umfleet, 1978). Zero time points were obtained by mixing [γ-^{32}P]ATP with the unlabeled ATP, adding cross-linked acto-SF-1, and quenching in 5.4% trichloroacetic acid after an incubation time of 20 s. Total radioactivity (cpm) was carried out by incubating [γ-^{32}P]ATP with cross-linked acto-SF-1 for 30 min, adding unlabeled ATP, incubating for 20 s, and finally quenching in 5.4% trichloroacetic acid. In P_i burst experiments reaction mixtures were quenched directly in 22% trichloroacetic acid. Zero time points and total radioactivities were carried out as described previously (Barman et al., 1983). The trichloroacetic acid contained 1 mM NaH_2PO_4.

In the experiments reported here, the size of the amplitude of any transient phase was of crucial importance, and to obtain these, accurate values for product at zero time were essential. Whether or not there was a transient burst phase, back-extrapolation of the data to zero time gave a blank that agreed well with the experimentally obtained blanks. These blanks were identical with those found previously with experiments with SF-1 ([^{32}P]P_i content of the [γ-^{32}P]ATP = 1.5–3%).

Experimental Conditions. Except where otherwise stated, all experiments were carried out at 15 °C in 5 mM KCl, 2 mM magnesium acetate, 0.1 mM dithiothreitol, and 50 mM Tris, adjusted to pH 8 with acetic acid, with or without 40% ethylene glycol. Cross-linked acto-SF-1 solutions were freed from air bubbles by centrifugation at a low speed in a desk centrifuge for 2 min.

Results

Experiments in Water. The results of ATP chase and P_i burst experiments in 5 mM KCl at 10 μM ATP are illustrated in Figure 1. These were carried out on the same day with the same preparation of cross-linked acto-SF-1. It is noteworthy that in the P_i burst experiment no transient phase can be discerned. As expected, the steady-state rates obtained in the two experiments agreed well.

ATP chase and P_i burst experiments were also carried out at 0.15 M KCl. Under these conditions, it was difficult to interpret the ATP chase results; the amplitude was very low and its kinetics too rapid to be exploitable (curve not shown). However, there appears to be a small P_i burst of about 0.1 μM P_i, but it is less than 10% of the active site concentration (Figure 2).

A way of obtaining k_{cat} with SF-1 is to carry out single-turnover experiments [e.g., Bagshaw & Trentham (1973) and Biosca et al. (1984b)]. In these experiments one follows the formation of [^{32}P]P_i on the $1/k_{cat}$ time scale (i.e., SF-1 plus [γ-^{32}P]ATP reaction mixtures are quenched directly in acid). The kinetics of the decomposition of intermediates containing [γ-^{32}P]ATP to [^{32}P]P_i are first order and give k_{cat} directly. A single-turnover experiment with cross-linked acto-SF-1 in 5 mM KCl is illustrated in Figure 3. There is a small transient

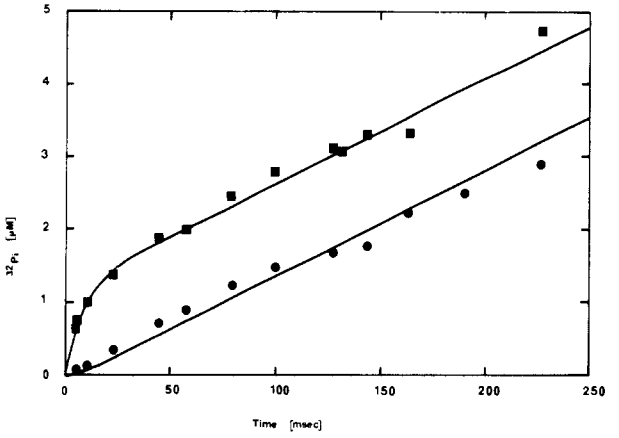

FIGURE 1: Time courses for the binding of ATP (■) and P_i burst (●) for cross-linked acto-SF-1 in water at 5 mM KCl. Reaction mixtures (2.5 μM SF-1 + 10 μM [γ-^{32}P]ATP) were quenched in unlabeled ATP or acid, and the [^{32}P]P_i was determined as described in the text. The continuous lines were computer simulated from Scheme II with $k = 70\ s^{-1}$, $k_{-2} = 0$, $k_3 = 13\ s^{-1}$, $k_{-3} = 10\ s^{-1}$, $k_4 = 80\ s^{-1}$, and active site concentration = 1.6 μM (see Treatment and Fitting of Data).

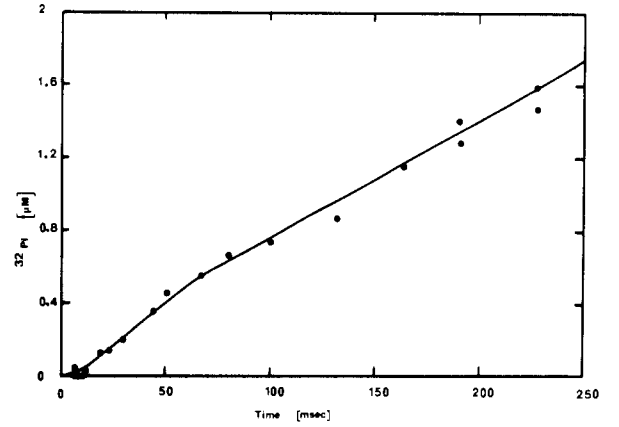

FIGURE 2: Time course for the P_i burst for cross-linked acto-SF-1 in water at 0.15 M KCl. The reaction mixtures were 2.5 μM SF-1 + 10 μM ATP. The continuous line was drawn by hand.

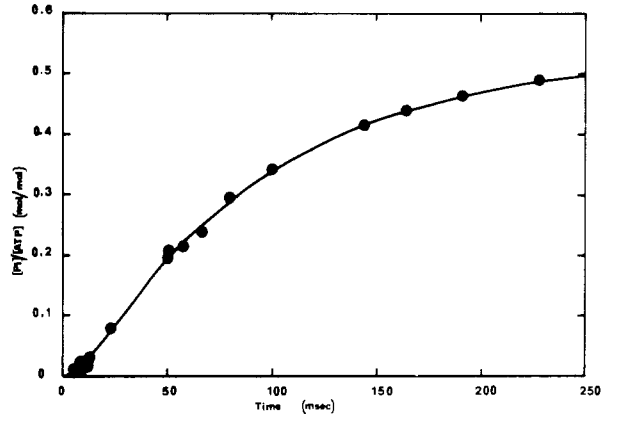

FIGURE 3: Single-turnover experiment with cross-linked acto-SF-1 in water at 5 mM KCl. The reaction mixtures were 9.3 μM SF-1 + 0.75 μM ATP. The continuous line was fitted to two exponentials with $k = 100$ (±10) s^{-1} for the first and 11.3 (±0.3) s^{-1} for the second and a final amplitude of 0.53 (±0.06).

lag phase (presumably a manifestation of the initial ATP binding process) followed by a slower, apparently first-order process with $k_{obsd} = 11.3$ (±0.3) s^{-1}. This agrees well with the k_{cat} calculated from multiturnover experiments under the same conditions: 10 (±2) s^{-1} (Figure 1); here, k_{cat} was obtained by dividing a steady-state by an ATP chase amplitude. This agreement confirms that one can titrate cross-linked acto-SF-1

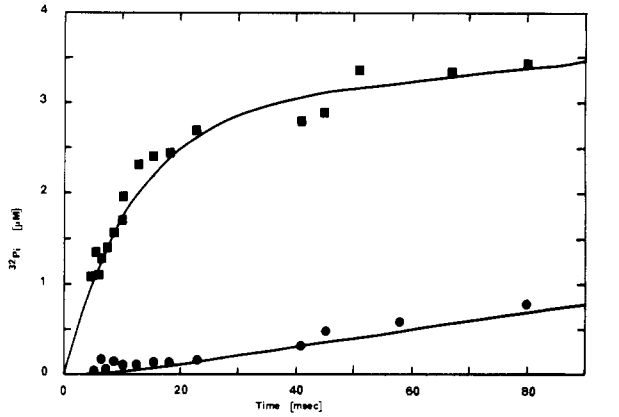

FIGURE 4: Time courses for the binding of ATP (■) and P_i burst (●) for cross-linked acto-SF-1 in 40% ethylene glycol at 5 mM KCl. The reaction mixture was 6 μM SF-1 and 30 μM ATP. The continuous lines were computer simulated according to Scheme II with the kinetic parameters in Tables I and II.

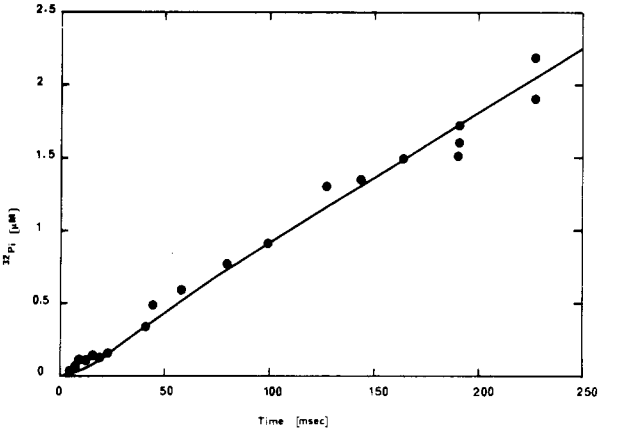

FIGURE 5: Extended time course for P_i burst with cross-linked acto-SF-1. For experimental conditions and procedures, see legend to Figure 4.

ATPase sites in ATP chase experiments.

On the time scale used, only about half of the [γ-^{32}P]ATP was hydrolyzed; all was hydrolyzed after an incubation time of several minutes. This result may be a manifestation of different ATP binding sites on cross-linked acto-SF-1 as has already been proposed for reversible acto-SF-1 (Biosca et al., 1984a). This problem was not further investigated here.

Experiments in 40% Ethylene Glycol. As indicated above, experiments with cross-linked acto-SF-1 in water were difficult to interpret, and further, they were complicated by instability problems. Therefore, we decided to continue our experiments with 40% ethylene glycol as the solvent as we had already done with SF-1 alone (Barman et al., 1983) and reversible acto-SF-1 (Biosca et al., 1984a). There is evidence that this solvent stabilizes the ATPase activity of cross-linked acto-SF-1 as prepared above (Rouayrenc et al., 1985).

The results of ATP chase and P_i burst experiments in 40% ethylene glycol and 5 mM KCl at 30 μM ATP are shown in Figure 4. The ATP chase curve is qualitatively very similar to those obtained with SF-1 alone (Barman et al., 1983) and reversible acto SF-1 (Biosca et al., 1984a); however, unlike with these materials, a P_i burst could not be discerned with cross-linked acto-SF-1. The P_i burst experiment was extended in time to 230 ms (Figure 5). The steady-state rates obtained from the ATP chase and P_i burst experiments were very similar.

The effect of the ATP concentration on the kinetics of its binding to cross-linked acto-SF-1 is illustrated in Figure 6;

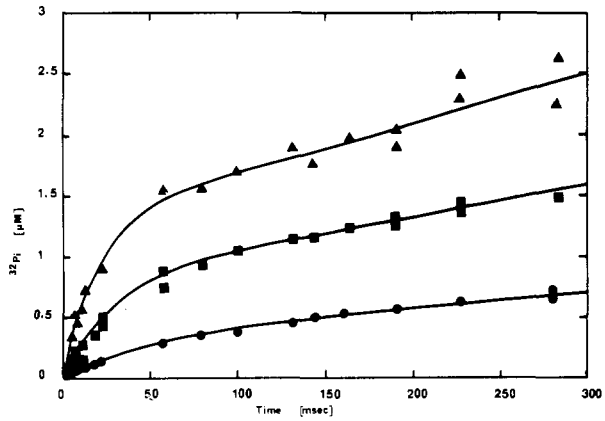

FIGURE 6: ATP chase experiments at different concentrations of ATP. The concentrations of ATP and SF-1 were respectively 5 μM + 1 μM (●), 10 μM + 2 μM (■), and 15 μM + 3 μM (▲). The continuous curves were computer simulated from Scheme II and the kinetic parameters of Tables I and II. For experimental details, see legend to Figure 4.

these experiments were carried out on the same preparation of cross-linked acto-SF-1.

Treatment and Fitting of Data. From our data, it was difficult to develop a kinetic scheme for cross-linked acto-SF-1. In particular, since in P_i burst experiments the amplitudes were nondetectable or very low, these contributed little information. Nevertheless, in aqueous solutions and at 0.15 M KCl there was a discernible transient P_i burst phase (Figure 2), which suggests an intermediate of the type AM·ADP·P_i (AM, cross-linked acto-SF-1). From our results, then, we do not have any reason to suppose that the initial steps for the binding of ATP to cross-linked acto-SF-1 are any different from those of SF-1. Thus, we have Scheme II, where the asterisks indicate different protein conformations. K_1 is a rapid equilibrium. This is a shortened version of the seven-step Bagshaw–Trentham pathway [e.g., Bagshaw et al. (1974)] for SF-1 that we used to interpret our results with reversible acto-SF-1 (Biosca et al., 1984a).

Scheme II

$$\mathrm{AM} + \mathrm{ATP} \overset{K_1}{\leftrightarrow} \mathrm{AM{\cdot}ATP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{AM^*{\cdot}ATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$$

$$\mathrm{AM^{**}{\cdot}ADP{\cdot}P_i} \xrightarrow{k_4} \mathrm{AM} + \mathrm{ADP} + \mathrm{P_i}$$

In ATP chase experiments, one determines the sum $([\mathrm{AM^*{\cdot}ATP}] + [\mathrm{AM^{**}{\cdot}ADP{\cdot}P_i}])k_{cat}/(k_{cat} + k_{-2})$ plus free $[P_i]$. In P_i burst experiments, one only determines $[\mathrm{AM^{**}{\cdot}ADP{\cdot}P_i}]$ plus $[P_i]$ [e.g., Barman et al. (1983)]. In both cases P_i is determined, and in each case the relevant integrated rate expression is the sum of two exponentials followed by a linear steady state (i.e., free P_i production). For details of the mathematical procedures used, see Capellos & Bielski (1972) and for myosin Taylor (1979). The two expressions were programmed on an Apple II 48K computer, and the parameters of Scheme II were adjusted to the experimental data by successive approximations.

A key assumption in the interpretation of ATP chase experiments concerns k_{-2}. Since the transient-phase amplitude $= k_{cat}/(k_{cat} + k_{-2})$ (Biosca et al., 1984a), these experiments only give significant transient burst phases if $k_{-2} \leq k_{cat}$. This was the case with SF-1 (Barman et al., 1983) and acto-SF-1 (Biosca et al., 1984a). With cross-linked acto-SF-1, the transient burst amplitudes in all of our experiments were ≥0.5 mol of P_i/mol of SF-1 protein (Figures 1, 4, and 6). With the parent SF-1 preparations, the chase amplitudes were in the range 0.63–0.70. If we assume that the SF-1 does not lose any of its ATPase activity during the cross-linking reaction, then $k_{-2} \leq 0.2k_{cat}$, i.e., $k_{-2} \leq 0.6$ s^{-1} in 40% ethylene glycol. In the fitting of our data, insignificant differences were obtained with $k_{-2} = 0.6$ s^{-1} or below. Therefore, since in any event k_{-2} is smaller than the other constants of Scheme II, we assumed in the final fitting that it is zero. Thus, the computer adjustment required the consideration of five parameters: k $(=k_2K_1[\mathrm{ATP}]/(1 + K_1[\mathrm{ATP}])$; k_3; k_{-3}; k_4; the ATPase active site concentration.

A way of facilitating the fitting process is to carry out ATP chase experiments at different ATP concentrations but with the same preparation of cross-linked acto-SF-1 (the active site to ATP concentrations ratio being constant). This was possible in 40% ethylene glycol. The set of constants obtained are given in Table I; the kinetic curves at five concentrations of ATP could be well fitted to these (results with 5, 10, and 15 μM ATP shown in Figure 6; that at 30 μM ATP in Figure 4; that at 20 μM not shown). We could not fit the data to other sets of constants (e.g., $k_3 \ll k_{-3}$ and $k_4 < k_3 + k_{-3}$). The effect of the ATP concentration on the kinetics of its binding to cross-linked acto-SF-1 could now be determined and is illustrated in Figure 7. The relationship is linear giving $K_1k_2 = 3.1\ (\pm 0.2) \times 10^6$ M^{-1} s^{-1}. We estimate $k_2 > 200$ s^{-1} and $K_1 < 1.6 \times 10^4$ M^{-1}.

Because of the difficulties indicated above, the experiments in water were difficult to exploit; the values given in Figure 1 in 5 mM KCl are only approximate. It was not possible to make any estimates for the kinetic constants from experiments in 0.15 M KCl.

Effect of Experimental Conditions on the Steady-State Parameters of Cross-Linked Acto-SF-1. In 40% ethylene glycol, the experimentally obtained K_m for ATP is 3 (±1) μM; this compares with a calculated value of 1.5 μM (Table II). Under the same experimental conditions, a value of 0.1 μM was found for SF-1 (Barman et al., 1983).

It was more difficult to determine the K_m for ATP in water. Here the initial rates were not always linear; this could be due to the instability of the ATPase site of the cross-linked acto-

Table I: Kinetic Constants for the Binding of ATP to Cross-Linked Acto-SF-1 in 40% Ethylene Glycol and 5 mM KCl at 15 °C[a]

prepn of cross-linked acto-SF-1	concn in reaction mixture: as SF-1 protein (μM)	concn in reaction mixture: ATP (μM)	kinetic constant[b]: k (s^{-1})	kinetic constant[b]: titration (ATP/SF-1, mol/mol)
1	1	5	15 (±2)	0.52 (± 0.05)
	2	10	30 (±3)	
	3	15	42 (±4)	
2	4	20	65 (±5)	0.50 (±0.05)
	6	30	90 (±10)	

[a] For other experimental conditions, see text. [b] The kinetic constants were obtained from Scheme II and computer simulation. $k = k_2K_1[\mathrm{ATP}]/(1 + K_1[\mathrm{ATP}])$, and titration = computer-fitted amplitudes of transient burst phases from ATP chase experiments (e.g., Figure 6). The values in parentheses are standard deviations. For the procedures used, see text.

Table II: Kinetic Constants for the ATPase Activity of Cross-Linked Acto-SF-1 in 40% Ethylene Glycol and 5 mM KCl at 15 °C[a]

k_1k_2 (M^{-1} s^{-1})	k_3 (s^{-1})	k_{-3} (s^{-1})	k_4 (s^{-1})	k_{cat} (s^{-1})	K_m (μM)
3.1 (±0.2) × 10^6	4 (±0.5)	4 (±2)	20 (±5)	3 (±0.3)	1.5 (±2)

[a] For other experimental conditions, see text. K_1k_2 was obtained from the ATP dependency of k (Figure 7), and other constants were obtained from Scheme II and computer fitting as described in the text. k_{cat} and K_m were calculated from the individual rate constants. The values in parentheses are standard deviations.

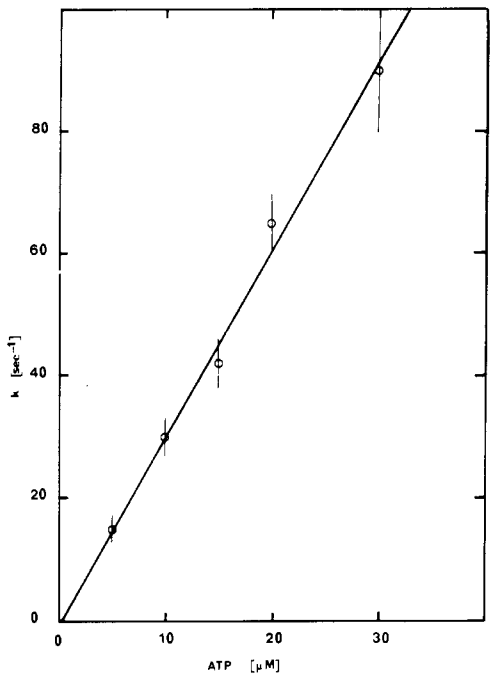

FIGURE 7: Dependence of the rate of binding of ATP (k) to cross-linked acto-SF-1 on the concentration of ATP in 40% ethylene glycol and 5 mM KCl. The data are from Table I; the continuous line was linearly computer fitted giving a slope of 3.1 (±0.2) × 10^6 M^{-1} s^{-1}.

Table III: Effect of Experimental Conditions on k_{cat} of Cross-Linked Acto-SF-1 and SF-1[a]

conditions	k_{cat} (s^{-1}) cross-linked acto-SF-1[b]	k_{cat} (s^{-1}) SF-1[c]
water, 5 mM KCl, 15 °C	10 (±2)	0.08
water, 5 mM KCl, 25 °C	42 (±4)	0.15
water, 0.15 M KCl, 15 °C	14 (±2)	0.08
40% ethylene glycol, 5 mM KCl, 15 °C	3.0 (±0.3)	0.06
40% ethylene glycol, 5 mM KCl, 25 °C	8.5 (±1)	0.11
40% ethylene glycol, 0.15 M KCl, 15 °C	4.0 (±0.5)	0.06

[a] Other experimental conditions, see text. [b] The values are from rapid flow quench or steady-state experiments and have been corrected for the active site concentration of the particular preparation used (see text). The values in parentheses are standard deivations. [c] From Barman et al. (1983) and Biosca et al. (1984b).

SF-1 at the concentrations used (Rouayrenc et al., 1985), but there may be other reasons (e.g., depolymerization of the F-actin). Although the values we obtained are only rough estimates, it appears that the K_m in water is higher than that in 40% ethylene glycol: about 10 μM in 5 mM KCl and 50 μM at 0.15 mM KCl.

The k_{cat} of cross-linked acto-SF-1 (i.e., at high ATP concentrations) was measured under various experimental conditions, and the results obtained are summarized in Table III. In 40% ethylene glycol, the values obtained agree well with those from ATP chase and P_i burst experiments at low ATP concentrations.

The 40% ethylene glycol decreases the k_{cat} of cross-linked acto-SF-1 4.5-fold. A similar decrease in k_{cat} was found for reversible acto-SF-1 (Travers & Hillaire, 1979) and also for two other ATP-handling enzymes—arginine kinase (Travers et al., 1978) and creatine kinase (Travers et al., 1979). The Mg^+-ATPase activity of SF-1 is insensitive to 40% ethylene glycol (Travers & Hillaire, 1979; Barman et al., 1983).

Discussion

Physical and Structural Properties of Cross-Linked Acto-SF-1. Although it is not our intention to fully discuss these here, some comments are necessary. First, because of the viscosity of its solution, cross-linked acto-SF-1 is difficult to handle in rapid reaction equipment. Our experiments were therefore limited to rather low concentrations in SF-1. Stopped-flow experiments are hard because of translucency problems. Second, so far a homogeneous preparation of a particular cross-linked acto-SF-1 complex has been difficult to prepare. As revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, cross-linked acto-SF-1 preparations consist mainly of a doublet band that is due to the two actin SF-1 dimers (i.e., one SF-1 molecule linked either by its 20K or 50K domain to one actin monomer in F-actin) together with higher polymers and unreacted actin (Mornet et al., 1981a; Sutoh, 1982, 1983; Greene, 1984; Heaphy & Tregear, 1984). In addition, preparations consistently contain a component (less than 8% of the total SF-1) that migrates in the same position as the SF-1 heavy chain (95K) and actin dimer. This component could not be removed by repeated washings of the cross-linked acto-SF-1 in pyroposphate buffers nor by several depolymerization–polymerization cycles (Rouayrenc et al., 1985).

Despite these difficulties, however, it was important to make an attempt at elucidating the reaction mechanism of cross-linked acto-SF-1. Because of the rapidity of the overall hydrolysis of ATP, high K_m, and the relative instability of cross-linked acto-SF-1 in water, the bulk of the work reported here was carried out in 40% ethylene glycol.

Binding of ATP to Cross-Linked Acto-SF-1. The results of the ATP chase experiments carried out in 40% ethylene glycol strongly suggest that when covalently bound to actin, SF-1 retains its ability to bind ATP tightly. Thus, by assuming $k_{-2} \ll k$, our data could be satisfactorily fitted to Scheme II at all of the concentrations of ATP used.

However, unlike with SF-1 (Barman et al., 1983) or reversible acto-SF-1 (Biosca et al., 1984a), we were unable to show an hyperbolic ATP dependency. Nevertheless, we consider that in common with SF-1 and reversible acto-SF-1 [for other enzymes, see Gutfreund (1972)] cross-linked acto-SF-1 binds ATP in two steps. Presumably, we could not attain the saturation plateau because of the prohibitively high concentrations of cross-linked acto-SF-1 needed for this. This problem is discussed for SF-1 in water (Barman et al., 1983). All we can say is that for cross-linked acto-SF-1 $k_2 > 200$ s^{-1} and $K_1 < 10^4$ M^{-1}.

The estimates for K_1 and k_2 of cross-linked acto-SF-1 are very different from the corresponding values for reversible acto-SF-1 and, in particular, SF-1 alone under identical conditions. This was not unexpected in view of the great sensitivity of these constants to the experimental conditions (Barman et al., 1983; Biosca et al., 1983, 1984b).

We note that the second-order rate constants (K_1k_2) for ATP binding to SF-1 alone (Barman et al., 1983), to reversible acto-SF-1 (Biosca et al., 1984), and to cross-linked acto-SF-1 (here) are very similar (2 × 10^6 M^{-1} s^{-1}, 1.8 × 10^6 M^{-1} s^{-1}, and 3.1 × 10^6 M^{-1} s^{-1}, respectively). Further, these values are close to the second-order rate constant for the dissociation of reversible acto-SF-1 by ATP (3.1 × 10^6 M^{-1} s^{-1}; Biosca et al., 1984a), and yet the individual constants involved in these processes are very different. It is difficult to come to any firm conclusion as to the significance of this similarity, which may, however, be fortuitous.

Although we do not have the same amount of data in water, our results in 5 mM KCl are qualitatively similar to those in 40% ethylene glycol. In particular, it appears that, as with SF-1 and reversible acto-SF-1, k_{-2} of cross-linked acto-SF-1

remains small. Now, Sleep & Hutton (1978) showed that k_{-2} for reversible acto-SF-1 is much larger than k_{-2} for SF-1 alone. Further, Marston & Tregear (1984) showed that AMPPNP is released much more rapidly from acto-SF-1 than from SF-1. We have already discussed this problem (Biosca et al., 1984a)—what is important in chase experiments is that $k_{-2} < k_{cat}$. For instance, when SF-1 is covalently linked to F-actin, k_{cat} increases several orders of magnitude and k_{-2} may do likewise but remains $< k_{cat}$.

The Cleavage Step. We next consider step 3 (the chemical step) of Scheme II and the following release of products step. In 40% ethylene glycol, unlike with SF-1 or reversible acto-SF-1 (i.e., at low actin concentrations) we could not discern a P_i burst with cross-linked SF-1 (Figure 4). This can be explained either by a small K_3 (i.e., a modification of the chemical step) or by $k_4 > k_3 + k_{-3}$. Now, as we discuss above, we adjusted the kinetic parameters of Scheme II to our experimental data so as to obtain the best fit and, thus, estimates of their values. We could not fit our data to a small K_3. The best fit was obtained with $K_3 = 1$ and $k_3 + k_{-3} = 8\ s^{-1}$. With SF-1 and under identical experimental conditions, $K_3 = 1$ and $k_3 + k_{-3} = 20\ s^{-1}$ (Biosca et al., 1984b); it appears, therefore, that the chemical step is not greatly changed in cross-lined acto-SF-1. A similar situation obtains with reversible acto-SF-1 [Biosca et al. (1984a) and references cited therein].

Our data could be fitted to $k_4 > k_3 + k_{-3}$. Thus, the value we estimate for k_4 for cross-linked acto-SF-1 is very much larger than that for SF-1: $20\ s^{-1}$ compared with $0.12\ s^{-1}$ (Biosca et al., 1984a). We conclude that the high value for the k_{cat} of the covalent complex is essentially due to a large increase in the rate of release of products (determined by k_4). Therefore, it appears that with cross-linked acto-SF-1 the chemical step is rate limiting. This is in accord with an energy of activation of its k_{cat} of about 80 kJ mol^{-1} (experiments at 15 and 25 °C, Table III): that for $k_3 + k_{-3}$ for SF-1 under the same conditions is 75 (±9) kJ mol^{-1} (Biosca et al., 1984b).

The curves fitted to the P_i burst show a small transient lag followed by a small transient burst phase. However, because of the high steady-state rates of cross-linked acto-SF-1, these transients were experimentally difficult to obtain. Further, any residual active and unbound SF-1 would hide any putative transient lag phase.

From our data, it is difficult to come to any precise conclusions as to the situation in water, but it does appear that in 5 mM KCl the P_i burst experiment (Figure 1) is qualitatively similar to that found in 40% ethylene glycol (Figure 4). Although it was difficult to fit the data in water to a unique set of constants, that given in Figure 1 suggests that, as in 40% ethylene glycol, $k_3 + k_{-3}$ for cross-linked SF-1 and SF-1 are similar: $23\ s^{-1}$ and $19\ s^{-1}$ (Barman et al., 1983), respectively. In 0.15 mM KCl there was a small but significant transient P_i burst phase (Figure 2), which, as for SF-1, can be explained by an increase in K_3 (Biosca et al., 1984b). Recently, Rosenfeld & Taylor (1984) report a small P_i burst with cross-linked acto-SF-1.

Our results are in accord with those of Webb & Trentham (1982), who showed that cross-linked SF-1 hydrolyzes ATPγS at the same rate as SF-1. With this analogue of ATP, the chemical step of the SF-1 reaction pathway is rate limiting. Further, the oxygen-exchange studies of Webb & Trentham (1982) with cross-linked acto-SF-1 and ATP show that $k_4 > k_{-3}$.

Recently, Stein et al. (1983) carried out P_i burst experiments with cross-linked acto-SF-1 in water and obtained relatively large transient P_i burst phases. This is in agreement with their previous work (Stein et al., 1981), with reversible acto-SF-1 at very high actin concentrations. To explain these results, Stein et al. (1984) suggest that there is a special rate-limiting step in the cycle following the ATP hydrolysis step. We are at present unable to explain these results, which are in variance with our own. A possible reason is that the catalytic properties of a particular cross-linked acto-SF-1 may depend on the composition of the medium used during the cross-linked reaction. There is some evidence for this from the work of Arata (1984) who carried out the reaction in the presence of different nucleotides and at different concentrations of KCl and obtained products with different ATPase activities.

Conclusions

Although the results given above suggest that covalently cross-linked acto-SF-1 can be used as a model for reversible acto-SF-1, the real situation may be more complicated than this. Does covalent acto-SF-1 mimic any particular intermediate on the cross-bridge cycle—in particular, the suggested actomyosin rigor complex? Any such comparison must take into account the recent suggestion of Greene (1984), namely, that most of the SF-1 bound in cross-linked acto-SF-1 is able to move quite freely. Thus, whereas the covalent cross-links prevent actomyosin dissociation when ATP is bound, there may be relatively large movements of the possibly flexible heads. Further, there is evidence that the structure of cross-linked acto-SF-1 could depend on the presence (and type) of nucleotide (ATP, ADP, AMPPNP) during the cross-linking reaction (Arata, 1984). Finally, from their oxygen-exchange studies, Shukla et al. (1983) show that with reversible acto-SF-1 at high actin concentrations the dissociative and nondissociative pathways (Scheme I) may be equally important.

Is there any relationship between our studies on covalently cross-linked acto-SF-1 and muscle contraction? Studies on acto-SF-1 suffer from the criticism that they involve only two of the several proteins implicated in muscle contraction. In particular, the regulatory system is absent—cross-linked acto-SF-1 hydrolyzes ATP without control. It is a system that, as it were, freewheels. Ferenczi et al. (1984) showed that the kinetics of ATP cleavage by skinned muscle fibers differ significantly from those obtained from solution studies. Very recently, Rouayrenc et al. (1985) showed that the regulatory system of skeletal muscle can modulate covalently cross-linked acto-SF-1. Therefore, it is important to carry out fast reaction experiments with cross-linked acto-SF-1 in the presence of the tropomyosin–troponin–Ca^{2+} system.

It was use of 40% ethylene glycol that allowed us to carry out our studies on SF-1, reversible SF-1, and now cross-linked acto-SF-1. Its use allowed us to compare the kinetics of the initial four steps of these three ATPases under identical conditions. First, ethylene glycol has the important effect of reducing the ATP binding kinetics to measurable levels (Barman et al., 1983). Second, it allowed for a detailed cryoenzymic study of SF-1 (Biosca et al., 1983, 1984b).

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Registry No. ATPase, 9000-83-3; 5′-ATP, 56-65-5; ethylene glycol, 107-21-1.

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