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Transient Kinetics of the Binding of ATP to Actomyosin Subfragment 1: Evidence That the Dissociation of Actomyosin Subfragment 1 by ATP Leads to a New Conformation of Subfragment 1†

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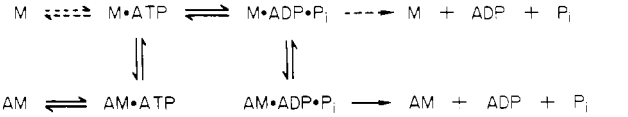
ABSTRACT: The initial steps by which ATP dissociates and binds to actomyosin subfragment 1 (acto-SF-1) were studied. Two techniques were used: stopped-flow (for acto-SF-1 dissociation kinetics) and rapid-flow quench with ATP chase quenching (for ATP binding kinetics). The experiments were carried out in 40% ethylene glycol–5 mM KCl, pH 8, at 15 °C. Under these conditions, the binding of SF-1 to actin remains very tight. As with SF-1, the ATP chase technique could be used, first, to titrate active sites and, second, to study the kinetics of ATP binding to acto-SF-1. The kinetic constants obtained were compared with those of SF-1 alone and with the acto-SF-1 dissociation kinetics under identical conditions. The kinetics of the acto-SF-1 dissociation did not vary with the actin to SF-1 ratio, but the ATP binding kinetics did, and a maximum value was reached at a mole ratio of 2.5. At high ATP (100 μM), k_{diss} = 300 s^{-1}, which compares with 49 s^{-1} and 13 s^{-1} for the ATP binding kinetics for acto-SF-1 (actin to SF-1 = 1:1) and SF-1, respectively. As with SF-1, the ATP binding to acto-SF-1 follows a hyperbolic law with the ATP concentration. This suggests a rapid equilibrium (K) followed by an essentially irreversible step (k). The values for K and k for the two proteins were significantly different: $K = 1.15 \times 10^5$ M^{-1} and k = 15.6 s^{-1} for SF-1, and $K = 3.6 \times 10^4$ M^{-1} and k = 62.7 s^{-1} for acto-SF-1. Taken together, these results show that the steps by which ATP dissociates and binds tightly to acto-SF-1 are kinetically distinct. Further, they imply that the conformation of SF-1 freshly released from acto-SF-1 by ATP is different from that of SF-1 alone. In the presence of ATP, this new structure then isomerizes to an intermediate (M*·ATP) which is also on the SF-1 ATPase pathway. Two possible mechanisms by which ATP dissociates and binds to acto-SF-1 are discussed. One of these involves one site for ATP on acto-SF-1. The other mechanism involves two sites: an acto-SF-1 dissociation site (where the ATP is not hydrolyzed) and an ATPase site (where the ATP is tightly bound and then hydrolyzed).

It is now well recognized that the various processes producing motility are intimately related to the actomyosin system. Thus, muscle contraction involves the sliding of interdigiting myosin and actin filaments. It is thought that this sliding process is driven by a cyclic interaction of myosin cross bridges (heads) with actin and coupled to ATP[1] hydrolysis. The ATPase sites are on the cross bridges, and movement is controlled by the various intermediates on the actomyosin ATPase pathway.

A model for actomyosin ATPase was first proposed by Lymn & Taylor (1971). In this, it is assumed that actin dissociates before hydrolysis occurs:



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

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Here, the actomyosin ATPase pathway (dissociative pathway) is indicated by the solid arrows; M is myosin and A actin. The top line refers to myosin ATPase. In this scheme therefore, the chemical step is common to myosin and actomyosin ATPases.

The results of recent work show that an additional step can be added to the Lymn & Taylor scheme. Thus, Stein et al. (1979) showed that at high actin concentrations the chemical step occurs without actin dissociation. Mornet et al. (1981) prepared covalently linked acto-SF-1 complexes with very high ATPase activities. These results show that the chemical step of acto-SF-1 ATPase can occur without actin dissociation, i.e., that the intermediate AM·ATP is transformed directly to AM·ADP·P_i. This has been termed the nondissociative pathway (Stein et al., 1979).

Extensive studies have been carried out on the acto-SF-1 pathway under dissociative conditions [for reviews, see Trentham et al. (1976), Taylor (1979), Adelstein & Eisenberg (1980), and Sleep & Smith (1981)], but there remain several crucial questions, in particular concerning the first steps. ATP binds essentially irreversibly to SF-1 in a two-step process (Chock, 1979; Taylor, 1979; Geeves & Trentham, 1982; Barman et al., 1983). Does acto-SF-1 also bind ATP in two steps and tightly? Also, does the dissociation of acto-SF-1 occur in the same step as the conformational change induced by ATP?

The ATP chase technique (Bagshaw & Trentham, 1973; Rose, 1980) leads directly to the kinetic constants for the binding of ATP to SF-1 (Barman et al., 1983). Its applicability depends on ATP being bound in an essentially irreversible process. In these studies, 40% ethylene glycol was used as the solvent; this reduces the binding constants to measurable levels and has allowed for cryoenzymic studies on SF-1 (Travers & Hillaire, 1979; Bêchet et al., 1979; Biosca et al., 1983, 1984; Millar & Geeves, 1983). Here, a similar study on acto-SF-1 was carried out. Under the conditions used, ATP binds tightly to acto-SF-1. The kinetics of the tight binding of ATP to acto-SF-1 was slower than the acto-SF-1 dissociation kinetics. However, it was faster than the kinetics of the tight binding of ATP to SF-1 alone. From these results, it appears that the SF-1 freshly released from acto-SF-1 by ATP has a modified structure.

Materials and Methods

Materials. Myosin and its subfragment 1 (SF-1) were prepared from rabbit muscle (Weeds & Taylor, 1975). Actin, also from rabbit muscle, was prepared following Spudich & Watt (1971). Protein concentrations were estimated at 280 nm by assuming $A^{1\%}_{1cm}$ = 7.5 for SF-1 (Wagner & Weeds, 1977) and 11 for actin (West et al., 1967). Molar concentrations were expressed in terms of an M_r of 110000 (Margossian & Lowey, 1978) for SF-1 and 42000 for actin (Collins & Elzinga, 1975). SF-1 active site concentrations were determined by ATP chase (Barman et al., 1983). [γ-^{32}P]ATP was obtained from Amersham International (Amersham, Bucks, United Kingdom).

Experimental Conditions. Except where otherwise stated, all experiments were carried out at 15 °C in 40% ethylene glycol, 5 mM KCl, 2 mM magnesium acetate, 0.1 mM dithiothreitol, and 50 mM Tris, adjusted to pH 8 with acetic acid. Air bubbles were removed from acto-SF-1 solutions by centrifugation at a low speed in a desk centrifuge for 2 min.

Quenched-Flow Experiments. The quenched-flow apparatus used have already been described (Barman et al., 1980). One is a rapid-flow quench apparatus (taking samples from 4 to 280 ms) and the other a time delay flow quench apparatus (times from 0.4 s upward). These devices were designed for mixing viscous solutions; with them, mixing was efficient with 40% ethylene glycol down to −20 °C. Both are thermostatically controlled, and all the experiments below were performed at 15 (±0.2) °C. Tight ATP binding to SF-1 or acto-SF-1 was followed by the ATP chase method, as described previously (Barman et al., 1983) with certain modifications. Typically, 1.1 mL of acto-SF-1 solution (1–20 μM) was mixed with 1.1 mL of [γ-^{32}P]ATP (5–200 μM) and after being aged was quenched in a 1000-fold molar excess of unlabeled ATP. The mixture was incubated for 30 s at 25 °C and quenched in 5.4% trichloroacetic acid, in water, containing 1 mM NaH_2PO_4, and the [^{32}P]P_i was determined (Reimann & Umfleet, 1978).

P_i burst experiments were carried out as above except that the reaction mixtures were quenched directly in trichloroacetic acid (22% in water containing 1 mM NaH_2PO_4). Zero time points and total radioactivities (cpm) of [γ-^{32}P]ATP solutions were carried out in triplicate as described previously (Barman et al., 1983). The precision of ATP chase and P_i burst experiments was critically dependent on the molar ratio of [γ-^{32}P]ATP to SF-1 and was kept within (5–10):1.

Stopped-Flow Experiments. These were carried out in a Union Giken RA 401 stopped-flow apparatus. The apparatus was modified to be thermostatically controlled to ±0.2 °C; the details of its use have been described (Hooper et al., 1983). The dissociation of acto-SF-1 by ATP was followed by the decrease in turbidity at 360 nm.

Turbidimetric Titration Experiments. The association of SF-1 and actin was studied by turbidity (White & Taylor, 1976) on an Aminco DW 2 spectrophotometer at 360 nm, which was thermostatically controlled to 15 (±0.2) °C.

Treatment of Data. A major objective of the present work was to carry out and interpret ATP chase and P_i burst experiments with acto-SF-1. This we had already done with SF-1 (Barman et al., 1983); here, the data could be satisfactorily fitted to the Bagshaw–Trentham scheme (Bagshaw & Trentham, 1973). As a first basis for an interpretation of the data obtained with acto-SF-1, we assume that the first

Table I: Kinetic Constants for Two Schemes for ATP Binding to Acto-SF-1 at 15 °C and Comparison with ATP Binding to SF-1[a]

	acto-SF-1		SF-1	
	constant	value[b]	constant	value
		Scheme II[c]		
	kK_T (M^{-1} s^{-1})	3.1 (±0.2) × 10^6		
	k_- (s^{-1})	<5		
	K'_1 (M^{-1})	2×10^5		
	k'_{-1} (s^{-1})	250		
	k'_1 (M^{-1} s^{-1})	5×10^7		
	k'_2 (s^{-1})	50		
		Scheme III		
dissociation site	kK_T (M^{-1} s^{-1})	3.1 (±0.2) × 10^6		
	k_- (s^{-1})	<5		
ATPase site	K'_1 (M^{-1})	3.6 (±0.6) × 10^4	K_1 (M^{-1})	1.15 (±0.22) × 10^5
	k'_2 (s^{-1})	62.7 (±6.4)	k_2 (s^{-1})	15.6 (±0.9)
	k'_{-2} (s^{-1})	<0.028	k_{-2} (s^{-1})	<0.01

[a] For experimental conditions, see text. [b] The values in parentheses are standard deviations. [c] Schemes II (one ATP site) and III (two ATP sites) refer to alternative mechanisms for the interaction of ATP with acto-SF-1. For convenience and comparative purposes the ATP binding constants for SF-1 are included under Scheme III.

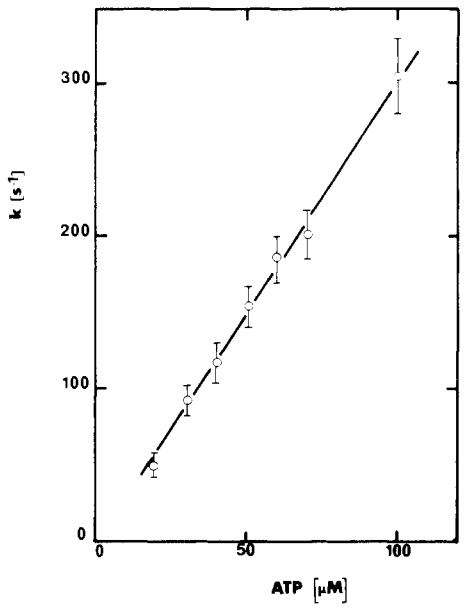


FIGURE 1: Dependence of the rate of dissociation of acto-SF-1 on ATP concentration. The decrease in turbidity on mixing acto-SF-1 with increasing concentrations of ATP was followed at 360 nm in a stopped-flow apparatus. The reaction mixture was 6 μM actin and 6 μM SF-1; for other details, see legend to Figure 2 and the text.

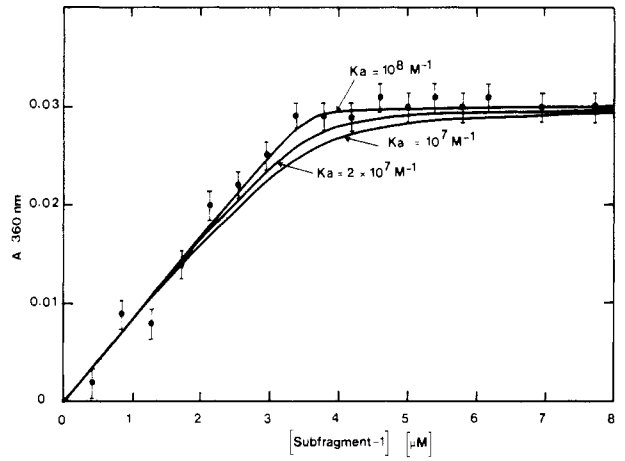


FIGURE 2: Turbidimetric titration of actin with SF-1. The actin concentration was 3.6 μM, and the experimental conditions were 40% ethylene glycol, 5 mM KCl, 2 mM magnesium acetate, 0.1 mM dithiothreitol, and 50 mM Tris adjusted to pH 8.0 with acetic acid. The temperature was 15 (±0.2) °C. The continuous curves were computer simulated by using K_a = [acto-SF-1]/([SF-1–acto-SF-1][actin–acto-SF-1]). For further details, see the text.

steps of acto-SF-1 and SF-1 ATPases are identical (see Scheme I). M is acto-SF-1 or SF-1; the asterisks indicate different protein conformations, and K_1 is an association constant (k_1/k_{-1}). In the following, the dissociation of acto-SF-1 by ATP is not taken account of.

Scheme I

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

Scheme I has two key features. First, k_{ON} for ATP [i.e., $k = K_1k_2[ATP]/(1 + K_1[ATP])$ is $\gg k_{cat}$, and second, the ATP on M*·ATP is hydrolyzed more rapidly than it is desorbed [i.e., $k_{OFF}(k_{-2}) < k_{cat}$]. These features allow for ATP chase experiments with SF-1 [e.g., Bagshaw & Trentham (1973), Taylor (1979), Chock et al. (1979), and Barman et al. (1983)], and here they were carried out with acto-SF-1. Such experiments are carried out on two time scales. Thus, SF-1 or acto-SF-1 plus [γ-^{32}P]ATP reaction mixtures milliseconds old (i.e., $t \ll 1/k_{cat}$) are quenched in a large molar excess of unlabeled ATP. The mixtures are incubated for several turnovers (i.e., $t \gg 1/k_{cat}$), and the [^{32}P]P_i is assayed after a second quench in acid.

The formation of [^{32}P]P_i is followed on the first time scale (i.e., $t \ll 1/k_{cat}$), and by extension of the treatment of Barman et al. (1983), it can be shown that

$$\frac{[[^{32}P]P_i]}{[M]_0} = \frac{[M^*{\cdot}ATP] + [M^{**}{\cdot}ADP{\cdot}P_i]}{[M]_0} = \frac{k_{cat}}{k_{cat} + k_{-2}}(1 - e^{-kt})$$

where $[M]_0$ is the initial active site concentration of SF-1 or acto-SF-1 ($<[ATP]_0$), $k = k_2K_1[ATP]_0/(k_1[ATP]_0 + 1)$, and $k_{cat} = k_4K_3/(1 + K_3)$.

The kinetics of the increase of [^{32}P]P_i with time is therefore first order; they lead to K_1 and k_2 regardless of the ratio of k_2 to $k_3 + k_{-3}$. At $t >> 1/k$, an amplitude of $k_{cat}/(k_{cat} + k_{-2})$ is reached. The size of this amplitude is critically dependent on the ratio of k_{cat} to k_{-2}. With SF-1, $k_{cat} >> k_{-2}$, the amplitude = $[M]_0$, and ATP chase experiments are a means of titrating the SF-1 catalytic site concentration [e.g., Geeves & Trentham (1982) and Barman et al. (1983)].

In P_i burst experiments, one determines [M**·ADP·P_i] with time. The extraction of rate constants from the data may or may not be a simple matter, depending on the ratio k to $k_3 + k_{-3}$. Thus, if $k >> k_3 + k_{-3}$, $k_{obsd} = k_3 + k_{-3}$, and if $k << k_3 + k_{-3}$, $k_{obsd} = k$. However under conditions where $k \sim k_3 + k_{-3}$, there is a transient lag phase of P_i, and to obtain $k_3 + k_{-3}$, computer simulation has to be resorted to. This problem has been discussed with SF-1 (Barman et al., 1983).

In turbidimetric titration experiments, one studies the interaction of a monomer protein, SF-1, with a filamentous polymer, F-actin, but as pointed out by White & Taylor (1976), the association can be treated as the independent binding of SF-1 to G-actin residues. Thus

$$\text{actin} + \text{SF-1} \underset{k_-}{\overset{k}{\rightleftharpoons}} \text{acto-SF-1}$$

with $k/k_- = K_a$. K_a was determined from turbidimetric titration experiments (White & Taylor, 1976). In these experiments, one adds increasing amounts of SF-1 to a solution of actin, and the acto-SF-1 formed is determined at 360 nm.

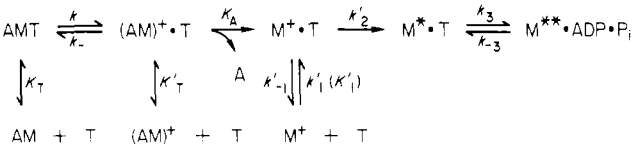
In dissociation experiments, acto-SF-1 is treated with increasing concentrations of ATP and the decomposition of acto-SF-1 followed by the decrease in turbidity at 360 nm in a stopped-flow apparatus. The dissociation of acto-SF-1 by ATP is at least a two-step process (Eccleston et al., 1976; Hofmann & Goody, 1978; Marston & Taylor, 1980; Geeves & Gutfreund, 1982):

$$\text{acto-SF-1} + ATP \underset{K}{\rightleftharpoons} \text{acto-SF-1}{\cdot}ATP \underset{k_-}{\overset{k}{\rightleftharpoons}} \text{actin} + \text{SF-1}{\cdot}ATP$$

At ambient temperatures, the variation of k_{diss} with ATP is linear. Thus under these conditions, [ATP] > $1/K$, and $k_{diss} = Kk[ATP] + k_-[actin]$. A plot of k_{diss} with ATP is linear with slope Kk (Figure 1).

Data Fitting. Data fitting and simulations were carried out on an Apple II 48K computer with the KINFIT program of Knack & Röhm (1981). This program allows for the calculations of standard deviations.

Scheme II[a]



[a] K_T, K'_T, K'_1, and K_A are association constants describing rapid equilibria, T is ATP, A is actin, and AM etc. are acto-SF-1 complexes.

The data from stopped-flow experiments were treated directly on a Sord Mark III microcomputer, which was directly connected to the Union Giken stopped-flow apparatus. This procedure allows, first, for the storage of kinetic curves and thus the accuracy of the experiment and, second, for the direct fitting of the data. Each point in Figure 1 is an average of eight experiments.

Results

Effect of 40% Ethylene Glycol on the Structure of Acto-SF-1. Limited tryptic digestion is a sensitive structural probe for SF-1 and acto-SF-1 (Mornet et al., 1979, 1981b). Thus, with SF-1 two bonds are cleaved and fragments of M_r 27K, 50K, and 20K result. With acto-SF-1, the 50K–20K joint is protected, and only two fragments of M_r 27K and 70K are produced.

SF-1 and acto-SF-1 were treated with trypsin in the presence and absence of 40% ethylene glycol, and the cleavage fragments were separated by electrophoresis following Mornet et al. (1979). The cleavage of the sensitive bonds of SF-1 and acto-SF-1 was somewhat slower in 40% ethylene glycol than in water, but the relative rates of cleavage of the two bonds of SF-1 were not modified. Ethylene glycol did not lead to the cleavage of additional bonds with either protein. It is concluded that the peptide bonds bordering the domains of SF-1 and acto-SF-1 (Mornet et al., 1979, 1981b) are in similar environments in 40% ethylene glycol and water.

The binding of SF-1 to actin was studied by turbidimetric titration (White & Taylor, 1976), and under the conditions used, $K_a > 5 \times 10^7$ M^{-1} (Figure 2). This high value explains the lack of variation of the amplitudes of the stopped-flow experiments with ATP. Therefore, with 1–10 μM acto-SF-1 (actin to myosin = 1) as used below, at least 87% saturation is ensured.

Acto-SF-1 Dissociation: Effect of ATP and Actin Concentrations. With [acto-SF-1] = 6 μM ([actin] = [SF-1]), the ATP dependency curve was linear up to at least 300 s^{-1}, and a plateau was not attained (Figure 1). The slope gives a second-order constant of 3.1 (±0.2) $\times 10^6$ M^{-1} s^{-1}; in water, Chock et al. (1976) give 2.4×10^6 M^{-1} s^{-1}. Sleep & Taylor (1976) give k_{diss} = 350–400 s^{-1} at 100 μM ATP in water and at 3 °C; this compares with our value of 300 s^{-1} in 40% ethylene glycol and at 15 °C.

The actin to SF-1 ratio had little effect on the dissociation process at a given ATP concentration. Thus, at 30 μM ATP with actin to SF-1 = 1:1, k_{diss} = 82 (±8) s^{-1}; at 2:1, k_{diss} = 85 (±7) s^{-1}; and at 3:1, k_{diss} = 79 (±8) s^{-1}.

Binding of ATP to Acto-SF-1. The results of ATP chase experiments with SF-1 and acto-SF-1 are compared in Figure 3. These were carried out with the same SF-1 preparation. With each there was a transient phase of bound ATP followed by the steady state of ATP hydrolysis. The amplitudes of the phases were very similar: 0.76 mol of ATP bound/mol of protein for acto-SF-1 and 0.72 for SF-1. Thus, it appears that for both SF-1 and acto-SF-1 the desorption of ATP (i.e., k_{OFF}) is slower than its hydrolysis (i.e., k_{cat}). The ATP chase method can, therefore, be used to titrate acto-SF-1 active sites. This result is in agreement with Sleep & Taylor (1976), Johnson & Taylor (1978), and Geeves & Trentham (1982).

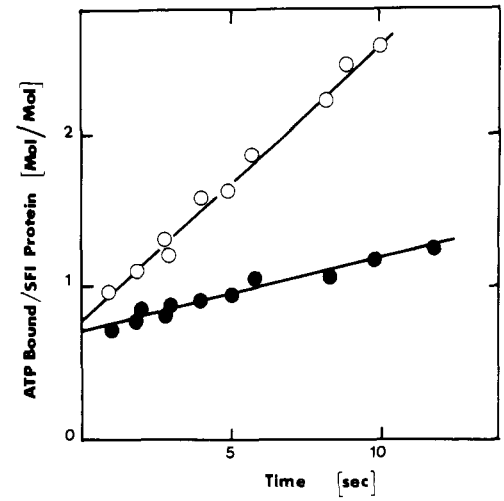


FIGURE 3: Titration of ATPase sites of SF-1 and acto-SF-1. The reaction mixtures were 10 μM SF-1 without (●) or with (○) 10 μM actin and 50 μM [γ-^{32}P]ATP. The reaction mixtures were quenched in 50 mM unlabeled ATP at the times indicated, and the [^{32}P]P_i was determined as described in the text. On the time scale used, the extrapolations of the steady states to zero time give the active site to SF-1 protein ratios as 0.72 mol/mol for SF-1 and 0.76 mol/mol for acto-SF-1. For experimental details, see legend to Figure 2.

Sleep & Hutton [1978; also see Sleep (1981)] show that the rate of desorption of ATP from acto-SF-1 varies with the actin concentration. This implies that the desorption of ATP from SF-1 and acto-SF-1 takes place by different pathways. At low concentrations of actin (as here), $k_{OFF} < k_{cat}$. From the steady states (Figure 3) and by assuming titration values of 0.76 mol of active site/mol of protein for acto-SF-1 and 0.72 for SF-1, the k_{cat} for acto-SF-1 = 0.25 s^{-1} and that for SF-1 is 0.064 s^{-1}.

A maximum value for k_{OFF} for ATP with acto-SF-1 can be estimated. Thus, if one assumes that SF-1 titrates 100%, then within the errors of the experiment acto-SF-1 titrates at least 90%; i.e., $k_{cat}/(k_{cat} + k_{OFF}) > 0.9$. With k_{cat} = 0.25 s^{-1}, k_{OFF} < 0.028 s^{-1} (at [actin] = 10 μM).

Kinetics of the Binding of ATP to Acto-SF-1. Three experiments were carried out at 100 μM ATP: acto-SF-1 dissociation and tight binding to acto-SF-1 and to SF-1 (Figure 4). The dissociation process [k = 300 (±30) s^{-1}] was considerably faster than the binding of ATP to acto-SF-1 [k = 49.2 (±6) s^{-1}], which, in turn, was faster than the binding to SF-1 [k = 13.2 (±1.9) s^{-1}].

The effect of the ATP concentration on the kinetics of its binding to acto-SF-1 and SF-1 is illustrated in Figure 5A. In all of these experiments the actin to SF-1 ratio was 1:1. A double-reciprocal plot with acto-SF-1 was linear (Figure 5B), which suggests that the binding process follows a hyperbolic law. Thus, as with SF-1, the binding of ATP to acto-SF-1 could be a two-step process.

The kinetics of the binding of ATP to acto-SF-1 appear to be first order, even at low concentrations of ATP where the dissociation kinetics are reasonably close to the binding kinetics. Transient lag phases or biphasic kinetics could not be discerned in any of the kinetic curves (e.g., Figure 6). With Scheme II as a model, the ATP binding constants for acto-SF-1 and SF-1 are significantly different (Table I).

Effect of Actin Concentration on the Tight Binding of ATP to Acto-SF-1. When the actin to SF-1 ratio was increased

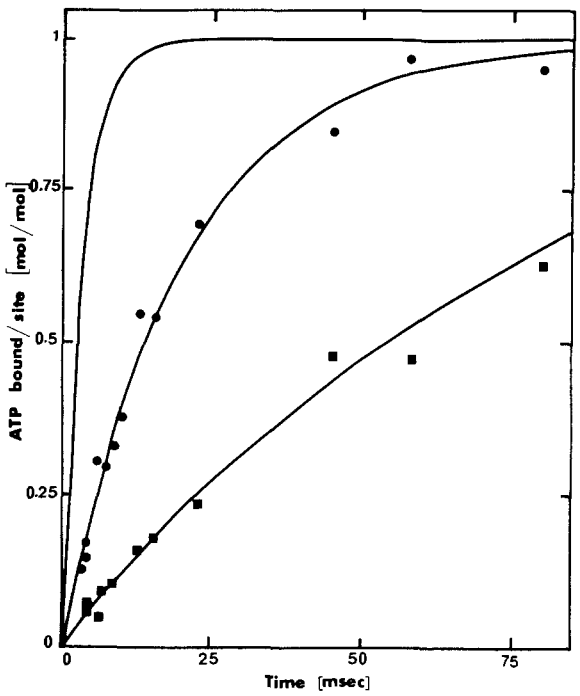


FIGURE 4: Time courses for acto-SF-1 dissociation and tight binding of ATP to acto-SF-1 and SF-1 at 100 μM ATP. The reaction mixtures were as follows: for acto-SF-1 dissociation (—), 6 μM SF-1 + 6 μM actin and 100 μM ATP; for tight ATP binding to acto-SF-1 (●), 10 μM SF-1 + 10 μM actin and 100 μM ATP; for tight ATP binding to SF-1 (■), 10 μM SF-1 and 100 μM ATP. The solid lines are computer-simulated curves. k_{obsd} for acto-SF-1 dissociation is 300 s^{-1} (expressed as fraction dissociated); k_{obsd} for tight ATP binding to acto SF-1 is 49 s^{-1} and to SF-1 is 13 s^{-1}. For experimental details, see the text.

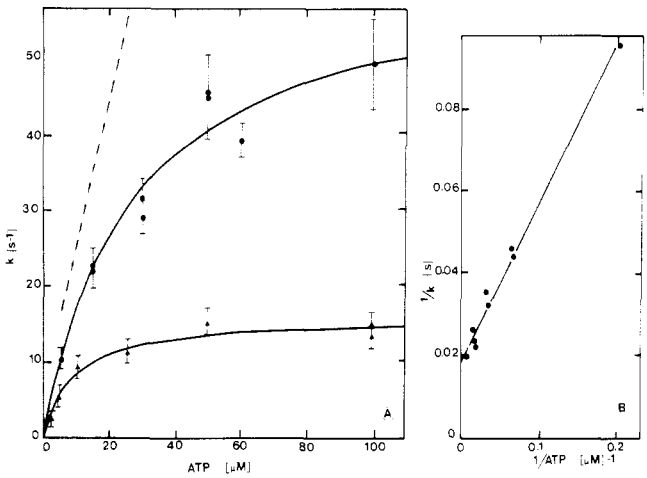


FIGURE 5: Dependencies of the rates of binding of ATP to acto-SF-1 and SF-1 on the concentration of ATP. (A) Plots of the variation of k with ATP for SF-1 (▲) and acto-SF-1 (●). The solid lines are from computer-simulated curves with, for SF-1, $K_1 = 1.15 \times 10^5$ M^{-1} and $k_2 = 15.6$ s^{-1} and, for acto-SF-1, $K_1 = 3.6 \times 10^4$ M^{-1} and $k_2 = 62.7$ s^{-1}. The ATP dependence for the dissociation of acto-SF-1 is shown by a broken line and is extrapolated from Figure 1. (B) Double-reciprocal plot of the variation of k with ATP for acto-SF-1. For experimental details, see the text.

at 15 or 30 μM ATP, the rate of the binding increased, and in each case a plateau was attained at a ratio of about 2–3 mol of actin/mol of SF-1 (Figure 7). Thus, whereas the actin to SF-1 ratio does not affect the ATP dissociation kinetics, the ATP chase kinetics are affected. The active site titration values obtained were within the range found for SF-1 alone; within the range used, the actin to SF-1 ratio does not appear to affect the ratio k_{cat} to k_{OFF}.

Attempts were made to obtain a saturation curve for the

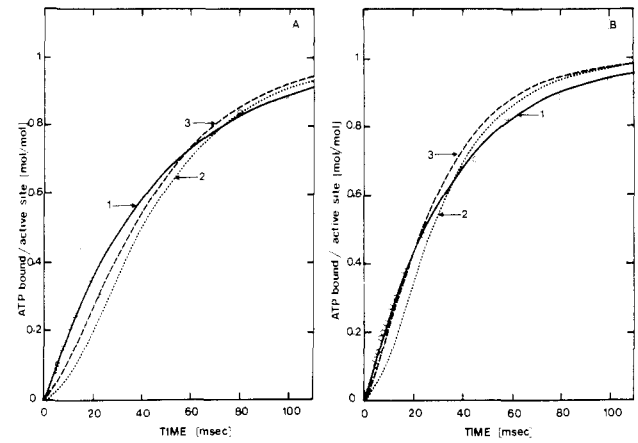


FIGURE 6: Time courses for the tight binding of ATP to acto-SF-1 at 15 μM ATP (A) or 30 μM ATP (B): (curves 1) experimental points fitted to a two-step process (e.g., Scheme I) with $k = 22$ s^{-1} for (A) and 29 s^{-1} for (B); (curves 2) experimental points fitted to Scheme II with K'_1 a rapid equilibrium, i.e., only $M^*_0 \cdot T$ assayed in cold chase (for constants used, see text); (curves 3) experimental points fitted to Scheme II with K'_1 not a rapid equilibrium, i.e., $M^*_0 + 0.2M_1 \cdot T$ assayed in cold chase (for constants used see text). Experimental conditions and procedures are given in the text.

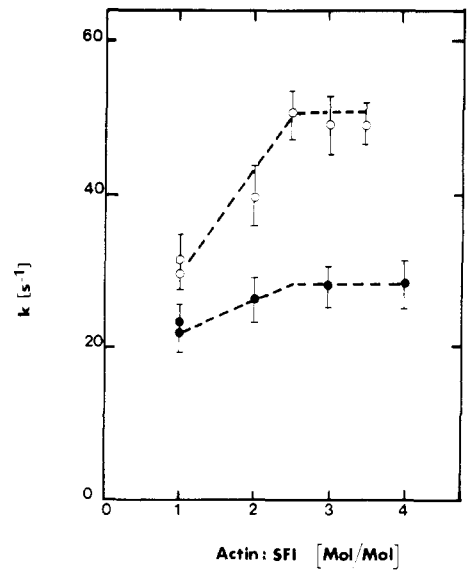


FIGURE 7: Dependency of the rate of binding of ATP to acto-SF-1 on the concentration of actin. This experiment was carried out at two ATP concentrations: 15 μM ATP (●) in reaction mixtures of 3 μM SF-1 plus increasing concentrations of actin (3–12 μM); 30 μM ATP (○) in reaction mixtures of 5 μM SF-1 plus actin (5–17.5 μM). For experimental conditions, see legend to Figure 1.

ATP binding kinetics with an actin to SF-1 ratio of 2.5 to 1, but because of viscosity problems, these were limited to 50 μM ATP with 12.5 μM actin and 5 μM SF-1. Higher ATP to SF-1 ratios seriously decrease the precision of the experiment. At 15 μM ATP, $k = 28.7$ (± 1.2) s^{-1}; at 30 μM ATP, $k = 50.0$ (±3.0) s^{-1}; at 50 μM ATP, $k = 74.3$ (± 7.7) s^{-1}. If one assumes a hyperbolic relationship, from these values "K_1" = 1 (±0.3) × 10^4 M^{-1} and "k_2" = 200 (±30) s^{-1} (see Scheme I).

Kinetics of the P_i Burst with Acto-SF-1. The results obtained with 50 μM ATP are illustrated in Figure 8. Since under these conditions k and $k_3 + k_{-3}$ are reasonable close, computer simulation had to be used to obtain $k_3 + k_{-3}$. The value obtained is given in Table II, where it is compared with that obtained with SF-1: 16.3 (± 1.7) s^{-1} for acto-SF-1 and 24.5 (±2.5) s^{-1} for SF-1. These values are reasonably close; this is in agreement with previous work, namely, that the

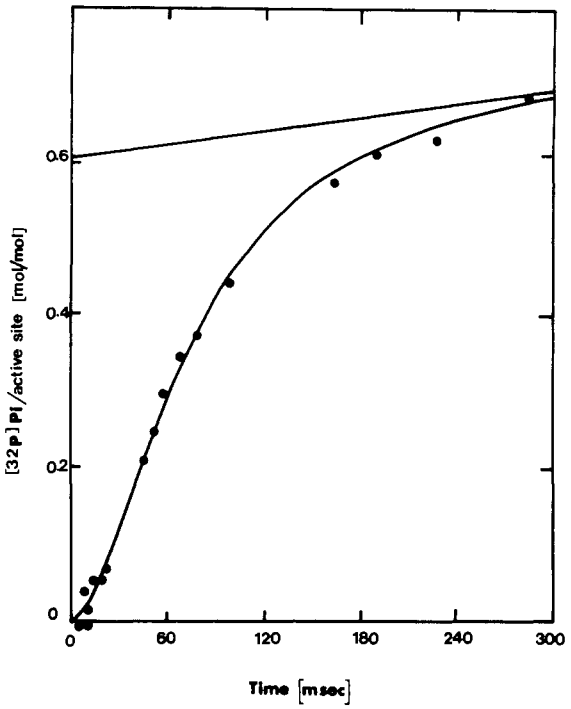


FIGURE 8: Time course for P_i burst of acto-SF-1 at 50 μM ATP. The reaction mixtures contained 5 μM SF-1 + 5 μM actin and 50 μM ATP. The solid curve is computer simulated with k_{chase} = 43 s^{-1} (Figure 5) and $k_3 + k_{-3}$ = 16.3 s^{-1}. The line refers to k_{cat} = 0.24 s^{-1}, extrapolated from experiments in the 1–10-s range. For experimental details and treatment of data, see the text.

Table II: Kinetic Constants for the Chemical and Rate-Limiting Steps of Acto-SF-1 and SF-1 (Scheme I) at 15 °C[a]

constant[b]	value[c] acto-SF-1[d]	SF-1
$k_3 + k_{-3}$ (s^{-1})	16.3 (±1.7)	24.5 (±2.5)
K_3	1.6 (±0.5)	2.1 (±0.5)
k_{cat} (s^{-1})	0.25 (±0.02)	0.064 (±0.006)
k_4 (s^{-1})	0.41 (±0.05)	0.094 (±0.010)

[a] For experimental conditions, see the text. [b] $k_3 + k_{-3}$ and K_3 were obtained from P_i burst experiments and computer simulations (Figure 8); k_{cat} was obtained from Figure 3, and k_4 was calculated from $k_4 = k_{cat}(1 + K_3)/K_3$. [c] The values in parentheses are standard deviations. [d] Acto-SF-1 used for $k_3 + k_{-3}$ and K_3 was 5 μM actin + 5 μM SF-1 and for k_{cat} was 10 μM actin + 10 μM SF-1.

kinetics of the chemical step are identical for SF-1 and acto-SF-1 (Lymn & Taylor, 1971; Johnson & Taylor, 1978; Stein et al., 1979; Geeves & Trentham, 1982). Further, this experiment confirms that the viscosity of the acto-SF-1 solution used does not present serious mixing problems with the rapid-flow quench device. The kinetic constants pertaining to the chemical and rate-limiting steps of acto-SF-1 and SF-1 are summarized in Table II.

Discussion

Effect of Ethylene Glycol on Acto-SF-1. Ethylene glycol at 40% was used as a solvent for two reasons. First, it allows for the determination of the temperature dependencies of the various steps on an enzyme pathway over a wide temperature range. This was done with SF-1 (Biosca et al., 1983, 1984). Second, it has the important effect of reducing the rapidity of the kinetics of the binding of ATP to SF-1 (Barman et al., 1983).

Whereas 40% ethylene glycol affects the relative values of the kinetic constants of the SF-1 pathway, the pathway itself does not seem to be modified (Bêchet et al., 1979; Travers & Hillaire, 1979; Barman et al., 1983). In ethylene glycol, actin activates the ATPase activity of SF-1 (Trentham, 1977; Travers & Hillaire, 1979). Millar & Geeves (1983), Marston (1982), and now ourselves show that acto-SF-1 is dissociated by ATP in ethylene glycol. Experiments on muscle fibres have been carried out in ethylene glycol (Tregear et al., 1982; Clark et al., 1980).

From the present, it seems that the susceptibilities of the peptide bonds of SF-1 and acto-SF-1 to trypsin are little affected by 40% ethylene glycol. Limited proteolysis is a sensitive probe for protein structure (Rupley, 1967), and this result is evidence that the conformations of SF-1 and acto-SF-1 are not greatly perturbed by 40% ethylene glycol. Bêchet et al. (1979) found that the optical rotatory spectra of SF-1 in water and 50% ethylene glycol were identical.

Further, with a $K_a > 5 \times 10^7$ M^{-1}, the binding of SF-1 to actin remains tight. The published values for K_a show a large variation [10^5–10^8 M^{-1}; e.g., Margossian & Lowey (1973), and Marston (1982)], which could be explained by the differences in the experimental conditions used and the difficulties in measuring high K_a values. Thus, K_a decreases sharply with the KCl concentration (Margossian & Lowey, 1973; Marston & Weber, 1975). Marston (1982) gives ($K_a = 10^8$ M^{-1} in water but 5×10^5 M^{-1} in 50% ethylene glycol. The latter is 100 times smaller than our estimate. This could be explained by the high ethylene glycol used (above 45% ethylene glycol, SF-1 and acto-SF-1 rapidly loose their ATPase activities; Travers & Hillaire, 1979), by the higher KCl (60 mM vs. 5 mM), and by the use of chemically modified proteins.

Dissociation of Acto-SF-1 by ATP Leads to a New State of SF-1. As shown in Figure 4, at a high ATP concentration (100 μM) the dissociation and binding kinetics of acto-SF-1 were very different: 300 s^{-1} and 49 s^{-1}, respectively. Thus, when ~80% of the acto-SF-1 has been dissociated, only ~20% of the total tight ATP binding has occurred to the freshly released SF-1. However, the tight ATP binding process occurred considerably more rapidly than with SF-1 alone (13 s^{-1}). This is a significant result; it implies that the conformation of SF-1 liberated from acto-SF-1 by ATP (which we call M^+) is different from that of SF-1 alone. Our finding is in agreement with the conclusions of Sleep & Taylor (1976), namely, that the cross-bridge cycle in muscle may require that the conformational states of the heads (SF-1) are different for detachment and attachment. This change of the SF-1 structure by actin has recently been discussed by Highsmith & Cook (1984); in particular, there is evidence that changes occur at the ATP binding site.

It appears, therefore, that when ATP interacts with acto-SF-1, the intermediate M^+·ATP is rapidly released and that on the time scale used, the kinetics of the isomerization of this complex to M·ATP (i.e., the adsorption complex between ATP and SF-1 alone) are slow. This slowness is not surprising; the reaction of actin with SF-1 is essentially irreversible, which implies that the M^+ structure (in acto-SF-1) returns to the original M structure (in SF-1 alone) slowly. Now, the closeness in the kinetics of the chemical steps of SF-1 and acto-SF-1 strongly suggest that the intermediate M*·ATP is common to both pathways. Thus, M^+·ATP isomerizes more rapidly to M*·ATP than to M·ATP, and we can write

$$AM + ATP \rightleftharpoons AM{\cdot}ATP \rightleftharpoons A + M^+{\cdot}ATP \rightarrow M^*{\cdot}ATP \rightleftharpoons M^{**}{\cdot}ADP{\cdot}P_i$$

The implication here is that the freshly released SF-1 "remembers" its actin conformation and reacts accordingly.

Effect of the Actin to SF-1 Ratio on the ATP Binding Kinetics. Whereas the kinetics of the acto-SF-1 dissociation

did not vary with the actin to SF-1 ratio, the ATP binding kinetics did, and a maximum rate was reached at a ratio of about 2.5 (Figure 7). This was an unexpected result, and an explanation was not immediately obvious. A banal explanation is that below a ratio of actin to SF-1 of about 2.5 the SF-1 is unsaturated in actin. However, in view of the titration experiment (Figure 2) and the concentrations of acto-SF-1 used, this seems unlikely.

The actin dependency could be caused by the SF-1 used being a mixture of the two SF-1 isoenzymes (Weeds & Taylor, 1975). The ATPase site of SF-1 is confined to the heavy chain [e.g., Taylor & Weeds (1977), Burke & Sivaramahrisnan (1981), and Sivaramahrisnan & Burke (1982)], but at low ionic strengths, as here, the two isoenzymes differ in the interaction with actin (Weeds & Taylor, 1975; Wagner et al., 1979; Geeves & Gutfreund, 1982). The two acto-SF-1 isoenzymes could bind ATP in different ways, but we found it difficult to fit such a situation to a satisfactory kinetic scheme for ATP binding to acto-SF-1.

A possible explanation comes from the work of Mornet et al. (1981a), who propose that the actin to SF-1 ratio influences the structure of the SF-1 in acto-SF-1. This proposal [but see Sutoh (1981)] is supported by the electron microscope studies of Amos et al. (1982). Different SF-1 structures could, therefore, be released from acto-SF-1 differing in their actin to SF-1 ratio, and these could bind ATP with different kinetics. Thus, it appears that the released SF-1 not only "remembers" its association with actin but also its particular environment in the acto-SF-1 complex. The solution to this problem must await further studies.

Interaction of ATP with Acto-SF-1: Possible Mechanisms. In order to give a minimum reaction scheme for the reaction of ATP with acto-SF-1, certain observations must be taken account of. First, the dissociation of acto-SF-1 takes place in at least two steps (Millar & Geeves, 1983). Second, the dissociation takes place before an apparently irreversible conformational change of SF-1. This leads to a key intermediate common to the SF-1 and acto-SF-1 pathways, M*·T. Finally, the intermediates preceding M*·T are in rapid equilibrium with ATP and the particular SF-1 structure involved (Scheme II).

In dissociation experiments, one follows the disappearance of all acto-SF-1 complexes, i.e., the appearance of M⁺·T, M⁺, and M*·T ($k_3 + k_{-3}$ is supposed to be small on the time scale used). In ATP chase experiments, it is supposed that only M*·T is assayed. Thus, when reaction mixtures (acto-SF-1 plus [γ-^{32}P]ATP) are quenched in unlabeled ATP, all radioactive complexes except M*·T are diluted out; to ensure this, the rapid equilibria K'_T and K'_1 are introduced. If, for example, M⁺·T were assayed in addition to M*·T, the ATP chase and dissociation kinetics would be identical, and this is not the case (Figure 4). It is unlikely that radioactive M*·T is rapidly diluted out to AM + T since the actin concentration is low and $k_- < 5$ s^{-1} (see below).

At high ATP concentrations, the dissociation and chase kinetics are clearly distinguishable and apparently first order (Figure 4). The kinetics of the dissociation (k_{diss}) varied linearly with the ATP concentration up to 100 μM, and the intercept on the abscissa was small (<5 s^{-1}; Figure 1). Therefore, $k_{diss} = kK_TT$, and from the slope, $kK_T = 3.1 \times 10^6$ M^{-1} s^{-1}. k is probably >600 s^{-1} and $K_T < 1 \times 10^4$ M^{-1}. A maximum value can be estimated for k_-. Thus from Figure 1, $(k_-)_{obsd} = k_-[A]K_A/(1 + [A]K_A) < 5$ s^{-1}. $(k_-)_{obsd}$ did not vary significantly with the actin concentration. Therefore, $[A]K_A >> 1$ and $k_- < 5$ s^{-1}.

The kinetics of the ATP chase (k_{chase}) varied little with the ATP concentration above 80 μM, and here, $k_{chase} = k'_2 \sim 50$ s^{-1} (Figure 5A). At high ATP concentrations, therefore, the dissociation and chase kinetics are satisfactorily explained by Scheme II. Scheme II predicts that at low ATP concentrations k_{diss} and k_{chase} become close, and in both cases the observed kinetics should be biphasic.

Transient lag phases in the kinetics of ATP chase experiments should be easy to discern. However, when ATP chase experiments were carried out at 15 and 30 μM ATP, transient lag phases were not obtained (Figure 6). Attempts were made to fit computer-simulated curves to the experimental data with Scheme II, the known values for $(k)_{obsd}$ and k'_2 (see above), and variable values for $(k_-)_{obsd}$ and K'_1. The closest fits obtained with $(k_-)_{obsd} = 5$ s^{-1} and $K'_1 = 2 \times 10^5$ M^{-1}, but distinct lags remained (Figure 6).

A key assumption in the interpretation of Scheme II is that in ATP chase experiments all the intermediates except M*·T are diluted out, i.e., that only radioactive M*·T is assayed. This may not be so. Thus, if K'_1 is not a rapid equilibrium (i.e., $k'_{-1} < k'_2$), some M⁺·T will be assayed together with M*·T. The amount of M⁺·T assayed critically affects the k_{chase} curves. Large amounts decrease the lag phase, but k_{chase} approaches k_{diss} rapidly as the amount of M⁺·T assayed increases. Decreasing the amount of M⁺·T assayed increases the lag phase. The best fit was obtained (Figure 6) with $K'_1 = 2 \times 10^5$ M^{-1} and the intermediates assayed = M*·T + 0.2M⁺·T. This gave $k'_{-1} \sim 5k'_2 \sim 250$ s^{-1} (since the intermediates assayed = M*·T + $[k'_2/(k'_2 + k'_{-1})][M^+\cdot T]$).

By making certain assumptions, all of the ATP chase data could be fitted reasonably well to Scheme II. The fits are not perfect but it is possible that they could be improved by further kinetic assumptions and by improvements in the precision of the assay method.

Another explanation for the ATP chase experiments stems from reports that there are at least two ATP sites per myosin head. Thus, the steady-state kinetics of SF-1 with ATP (Yee et al., 1980) or β-naphthyl triphosphate (Fujisaki & Asai, 1982) are nonlinear. Pyrophosphate (which dissociates acto-SF-1) inhibits SF-1 ATPase competitively and noncompetitively (Yee et al., 1980). Ribose 5-triphosphate binds to two sites on SF-1 (Eccleston, 1980). Meeusen & Cande (1979) modified HMM with *N*-methylmaleimide; the modified HMM had enhanced Ca^{2+}-ATP activity and bound normally to actin, but the corresponding acto-HMM complex was not dissociated by ATP.

A scheme involving two sites for ATP on acto-SF-1 (Scheme III) would explain all of the ATP chase experiments. The scheme supposes that there is a dissociative site (where the ATP is not hydrolyzed) and a site where the ATP is tightly bound and then hydrolyzed. The two processes are independent. For dissociation (reaction a), the asterisk indicates a different conformation of acto-SF-1. Estimates for kK_T and k_- were obtained from Figure 1 and are summarized in Table I. For tight ATP binding (reaction b), ATP binds to the ATPase site of an actin-modified conformation of SF_1, M⁺. This process occurs independently of the presence of actin. This scheme predicts that the tight ATP binding kinetics are hyperbolic. Estimates for K'_1, k'_2 (Figure 5), and k_{-2} are given in Table I.

Scheme III

$$AM + T \overset{K_T}{\rightleftharpoons} AMT \underset{k_-}{\overset{k}{\rightleftharpoons}} (AM^*)\cdot T \overset{K_A}{\rightleftharpoons} A + MT \quad \text{(a)}$$

$$M^+ + T \underset{K'_1}{\rightleftharpoons} M^+\cdot T \underset{k_{-2}}{\overset{k'_2}{\rightleftharpoons}} M^*\cdot T \quad \text{(b)}$$

A two-site scheme for acto-SF-1 ATPase is at once simple and accommodates our results. We are not aware of any contradictory published data. It is clear that in single-turnover experiments [i.e., [acto-SF-1] > [ATP]; e.g., Sleep & Hutton (1976) and Geeves & Trentham (1982)] there could be a competitive effect between the two putative sites for ATP. Any anomaly in ATP binding kinetics would depend on the relative values of the kinetic constants associated with the two sites under the particular experimental conditions used. It is not impossible that the findings of Sleep & Hutton (1976) (i.e., that under single-turnover conditions ATP is released much more rapidly from acto-SF-1 than from SF-1) can be explained by a two-site scheme. It is not possible to come to a final decision as to mechanism of the interaction of ATP with acto-SF-1. An answer to this must await the results of further experiments.

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Registry No. ATP, 56-65-5; ATPase, 9000-83-3.

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Interaction of Fluorescent Adenine Nucleotide Derivatives with the ADP/ATP Carrier in Mitochondria. 1. Comparison of Various 3′-*O*-Ester Adenine Nucleotide Derivatives†

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ABSTRACT: Fluorescent 3′-*O*-acyl-substituted adenine nucleotide (dimethylamino)naphthoyl and trinitrophenyl groups were studied for binding to the ADP/ATP carrier in mitochondria and submitochondrial particles. The changes in fluorescence intensity and emission maximum are for the most part similar to those observed in nonaqueous solvents. The (dimethylamino)naphthoyl derivatives from a largely quenched aqueous state have a shortwave shift up to 85 nm and increase up to 90-fold (1,5 derivative), whereas the little quenched naphthoyl derivatives show a fluorescence decrease and the weakly fluorescent trinitrophenyl derivative shows only a small increase on binding. All derivatives are good inhibitors (K_I = 1–10 μM) of nucleotide transport. The fluorescence titrations have an apparent $K_{1/2}$ = 2–7 μM. The fluorescence of the 1,5-DAN nucleotide is fully suppressed by bongkrekate but only partially suppressed by carboxyatractylate. The fluorescence response is much stronger in submitochondrial particles than in mitochondria. Both facts suggest fluorescent binding to the "m" state of the carrier site at the inner face of the membrane. 1,5-DAN-AMP shows a slightly more efficient binding than DAN-ADP or DAN-ATP.

Binding studies to the ADP/ATP carrier of mitochondria have proved to be a treasure house full of information elucidating modes of carrier function (e.g., Klingenberg et al., 1972; Weidemann et al., 1970; Klingenberg, 1976). Due to the inherent changes in the binding center of a carrier, considerable information of the functional state of the carrier can be revealed by both static and dynamic binding studies. These have so far been based on isotopic-labeled ligands, and therefore kinetic studies have been difficult. Ligands with fluorescent reporter groups have been sought, which might reflect important conformational changes relative to transport. Neither the substrates ADP and ATP nor the tightly binding inhibitors ATR[1] and BKA have an optical or fluorescent signal that can be monitored in binding studies. Because of the high selectivity of the carrier binding center, well-known fluorescent derivatives of ADP or ATP such as the ethenoadenine or 2-aminopurine nucleotides are nonbinding (Graue & Klingenberg, 1979). Only the structurally closely related and fluorescent adenine isomers, formycin di- and triphosphate, interact with the ADP/ATP carrier However, the characteristics of this fluorescence are rather disadvantageous such as low quantum yield, very short fluorescence lifetime (0.8 ns), and a strong overlap of excitation and emission with the signals of tryptophan. In part these difficulties were overcome by fluorescence transfer from formycin to anthracene derivatives (Graue & Klingenberg, 1979).

It was therefore greatly welcomed when Schäfer & Onur (1980) observed that (dimethylamino)naphthoyl esters of ADP etc. bind to the nucleotide carrier in submitochondrial particles, exhibiting a strong fluorescence signal. Before employing and exploring the binding possibilities of this compound to the ADP/ATP carrier on a broader scale, we had to develop a chemical synthesis of the most interesting substituent, 5-(dimethylamino)-1-naphthoic acid. The studies reported here concentrate on the carrier still located in the mitochondrial membrane. A part of these results has been reported previously (Klingenberg, 1981).

Materials and Methods

Chemical Synthesis. N^6-(2,4-Dinitrophenyl)adenosine 5′-triphosphate (DNP-ATP) was synthesized at a 35% yield by the method of Hiratsuka et al. (1973) with the modification that the reaction was allowed to proceed for 48 h and two Sephadex LH-20 (3 × 80 cm) purification steps were employed. 2′(3′)-*O*-(2,3,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was synthesized at a 62% yield by the method of Hiratsuka & Uchida (1973) also with the modification that two Sephadex LH-20 (3 × 90 cm) steps were employed and that fractions containing homogeneous TNP-ATP were pooled and immediately adjusted to pH 7 with Tris base; the Meisenheimer complex undergoes a slow hydrolysis process, forming ATP and picric acid at −20 °C, necessitating

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[1] Abbreviations: ATR, atractylate; BKA, bongkrekate; BHM, beef heart mitochondria; BHSMP, beef heart submitochondrial particles; CAT, carboxyatractylate; DAN-AMP, [(dimethylamino)naphthoyl]-adenosine 5′-monophosphate; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; RLM, rat liver mitochondria; RLSMP, rat liver submitochondrial particles; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; ATPase, adenosinetriphosphatase.

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