Potentiated State of the Tropomyosin Actin Filament and Nucleotide-Containing Myosin Subfragment 1†

John M. Murray, Mary K. Knox, Cynthia E. Trueblood, and Annemarie Weber*

ABSTRACT: The main purpose of this study was to determine whether potentiation of acto-S-1 ATPase activity (activity higher than that obtained with tropomyosin-free actin) could be caused by nucleotide-containing acto-S-1 complexes. In addition, we wanted to know whether these complexes also have a positive cooperative effect on their own apparent binding constant under conditions where nucleotide-free acto-S-1 complexes cause potentiation of ATPase activity. Using calcium-saturated troponin–tropomyosin actin filaments, we observed potentiation of ATPase activity in the presence of 5.0 mM magnesium 5′-adenylyl imidodiphosphate (MgAMPPNP) and calculated that the ability of acto-S-1–AMPPNP complexes to cause potentiation must have been very similar to that of nucleotide-free acto-S-1 complexes. In extension of earlier studies, potentiated acto-S-1 ATPase activity was characterized by an increase in V_{max} and, as observed before, a lowering of the apparent K_M for subfragment 1 (S-1). Under conditions similar to those that produce the potentiation of acto-S-1 ATPase activity, the apparent actin binding constant of nucleotide-free S-1 was increased about 3–5-fold while the apparent binding constant of AMPPNP to actin-bound S-1 was reduced to (2.5–10) × 10^2 M^{-1} compared to that of about (1–5) × 10^3 M^{-1} for S-1 bound to tropomyosin-free actin. Under the same conditions, the apparent binding constant of S-1–AMPPNP to actin was not increased. We suggest that a potentiated state of the tropomyosin actin filament is produced by the cooperative action of acto-S-1 or acto-S-1–AMPPNP complexes. The potentiated state is characterized by an increase in the V_{max} of the acto-S-1 ATPase activity, increased binding constants for S-1 and S-1–ADP, and increased binding of tropomyosin to actin.

Tropomyosin is a constituent of all muscle thin filaments and is bound to some of the actin filaments of many nonmuscle cells. It plays an essential role in the regulation of those muscles in which the transition between contraction and relaxation depends on a change of state of the thin filament (Ebashi et al., 1969; Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973). Its role has not been understood in muscles where this regulation is accomplished by the myosin molecule directly or possibly indirectly through myosin phosphorylation.

Therefore, it is of interest that tropomyosin is capable of modifying the behavior of actin filaments that do not contain troponin (Murray et al., 1975, 1980a,b; Bremel et al., 1972). Tropomyosin, in the absence of troponin, modifies the activation of myosin ATPase by actin in two opposite ways: either by reducing this activation or by enhancing it. These two tropomyosin effects are called inhibition or potentiation of actomyosin ATPase. Inhibition occurs when the S-1 (subfragment 1)[1] to actin ratio is low, i.e., about one S-1 per 30 actin molecules (Murray et al., 1975, 1980a,b). Under these conditions, the presence of tropomyosin on the actin filament reduces the acto-S-1 ATPase activity by half (Eaton et al., 1975; Murray et al., 1975, 1980a,b).

Increasing the S-1 to actin ratio changes tropomyosin inhibition to tropomyosin-dependent potentiation of acto-S-1 ATPase activity, so that it is severalfold faster than the ATPase activity with tropomyosin-free actin. In other words, potentiation of the cofactor activity of actin requires both the presence of tropomyosin and a sufficiently high degree of saturation of the actin filaments with S-1. What may constitute a sufficiently high degree of saturation with S-1 will be discussed later.

Of the various intermediate acto-S-1 complexes that occur during acto-S-1 ATPase activity [for a review, see Taylor (1979) and Adelstein & Eisenberg (1980)], only rigor complexes (nucleotide-free acto-S-1 complexes) have been shown unequivocally to cause potentiated ATPase activity (Bremel et al., 1972). Although rigor complexes are postulated to be an intermediate during bridge cycling (Eisenberg & Greene, 1980), their steady-state concentration is expected to be low at physiological ATP concentrations. A number of years ago (Bremel et al., 1972), we described experiments which seemed to indicate that the nucleotide-containing acto-S-1 complexes which prevail at saturating ATP concentrations (possibly acto-S-1–ADP·P) also cause potentiation. Later, however, we learned that this interpretation of the experiments is not unambiguous. Even in the presence of saturating ATP levels, rigor complexes cannot be ruled out as the cause of potentiation unless the existence of ATP-resistant rigor complexes can be excluded. ATP-resistant rigor complexes are formed with S-1 whose ATP binding site is nonfunctional so that ATP can no longer dissociate the rigor complex (Pemrick & Weber, 1976). We have shown previously that such myosin is produced during treatment with sulfhydryl reagents (Barany & Barany, 1959; Pemrick & Weber, 1976), and we have evidence suggesting that each S-1 preparation contains this S-1 as a contaminant, possibly due to sulfhydryl oxidation by air (Murray et al., 1981). Therefore, it is difficult to be sure to what extent potentiation at saturating ATP concentrations is due to these rigor complexes rather than to one of the nucleotide-containing acto-S-1 intermediates. To minimize the ambiguities intro-

† From the Medical Research Council, Cambridge, England, and the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received April 16, 1981; revised manuscript received August 21, 1981.* This work was supported by a research grant from the National Institutes of Health (HL 15-692).

* Correspondence should be addressed to this author at the Department of Biochemistry and Biophysics, University of Pennsylvania.

[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; [^{3}H]NEM, *N*-[^{3}H]ethylmaleimide; S-1, myosin subfragment 1; S-1–ADP·P, the fluorescent S-1 complex with both ADP and P that is the first product of ATP hydrolysis; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-0906$01.25/0

duced by ATP-resistant rigor complexes, we determined whether S-1 complexed with the ATP analogue 5′-adenylyl imidodiphosphate (AMPPNP) can cause potentiation. We present experiments showing that acto-S-1 complexes containing the nucleotide AMPPNP are just as competent to cause potentiation of ATPase activity as are rigor complexes.

We characterized the potentiated state with respect to the binding of S-1. Measurements of binding can provide an estimate of how great an energy barrier separates the potentiated from the nonpotentiated state of the actin filament (we describe the actin filament as potentiated when actomyosin ATPase is potentiated). We compared S-1 binding to troponin–tropomyosin actin in the potentiated state with S-1 binding to tropomyosin-free pure actin and to troponin–tropomyosin actin in the nonpotentiated state. A method which permits one to obtain actin binding constants for nucleotide-free S-1 and S-1–AMPPNP is the titration of acto-S-1 with increasing concentrations of AMPPNP (Greene & Eisenberg, 1978; Murray et al., 1981). Transition to the potentiated state increased binding of nucleotide-free S-1 4–6-fold. By contrast, we did not measure an increase in the binding constant for S-1–AMPPNP.

Materials and Methods

Protein Preparations. All proteins were derived from the hind leg and back muscles of rabbits. *Myosin subfragment 1* (S-1) was prepared according to Lowey et al. (1969) with the modifications described previously (Murray et al., 1981). The main change in the preparation consisted of selecting the S-1 fraction that precipitated as a discrete fraction between 50 and 55% ammonium sulfate saturation and rejecting the fraction that precipitated between 60 and 65% saturation. The 50–55% fraction had a greater actin-activated ATPase activity than the 60–65% fraction (50–70% higher) and was less digested than the later fraction. However, there was not a really distinct difference in gel bands between the two fractions such as the appearance or disappearance of a band on $NaDodSO_4$ gels.

Actin was prepared according to Spudich & Watt (1971) with the modifications described recently (Murray et al., 1981). It was homogeneous on overloaded $NaDodSO_4$ gels except for an occasional small band just in front of actin but not completely separated from it. We are not sure whether this occasional, very minor contamination is a proteolytic actin fragment.

Troponin and tropomyosin were prepared as a complex by extraction of a lyophilized Ebashi powder (Ebashi & Ebashi, 1964) with 1 M KCl, 2 mM $MgCl_2$, and 10 mM Tris, pH 7.4, followed by centrifugation at 100000*g* to remove contaminating actin. *Troponin–tropomyosin actin filaments* were prepared according to Bremel and colleagues [1972; see also Murray et al. (1981)]. In a number of preparations, the troponin–tropomyosin actin filaments contained an unidentified contaminant from the Ebashi extract that caused increasing depolymerization of the troponin–tropomyosin actin with time.

Protein concentrations were measured with the Lowry reaction (Lowry et al., 1951) by using bovine serum albumin as the color standard and calculating the protein concentrations from tables for myosin and actin constructed after determination of the protein concentrations by Kjeldahl. *Zonal $NaDodSO_4$ gel electrophoresis* was carried out with disc gels according to Laemmli (1970) with fast green as a stain as described by Potter (1974).

Protein Modification. For a few experiments, myosin was labeled before proteolysis with *N*-[^{3}H]ethylmaleimide ([^{3}H]NEM) as recently described (Murray et al., 1981). In experiments using native S-1, actin was labeled at cysteine-373 with [^{3}H]NEM (Porter & Weber, 1979).

Reagents. AMPPNP was obtained from Boehringer and was routinely checked for purity before use both by thin-layer chromatography and by enzymatic measurements for a quantitative measurement of the contaminating ADP and ATP concentrations. The enzymatic measurements were performed with Boehringer kits containing the pyruvate kinase + lactic dehydrogenase system and the phosphoglycerokinase + phosphoglyceraldehyde dehydrogenase + triose isomerase + glycerophosphate dehydrogenase system for measurement of contaminating ADP and ATP concentrations, respectively. Contamination with ADP + ATP varied from 1 to 3 mmol/mol of AMPPNP during the S-1 binding experiments and was 1 mmol/mol of AMPPNP during the ATPase activity measurements.

ATPase Activity Determination. (1) ATPase Activity as a Function of Increasing S-1 Concentrations. For these experiments, S-1, present in a stock solution of 40 mg/mL, was weighed into every assay tube before adding the actin with the incubation mixture containing (in final concentrations) 10 mM imidazole, pH 7.0, 1.0 mM $MgCl_2$, and 1.0 mg/mL creatine kinase. The assay was started by the addition of a mixture of MgATP and creatine phosphate to give final concentrations of 1.0 and 5.0 mM, respectively. The assay was terminated with *p*-mercuribenzoate (final concentration 50 mM) and analyzed for creatine as previously described (Maruyama & Weber, 1972).

(2) ATPase Activity as a Function of Increasing ATP Concentrations. For these experiments with constant S-1 and actin concentrations, the proteins were mixed with imidazole buffer and creatine kinase as described above and 5.0 mM $MgCl_2$ in excess over MgAMPPNP concentration (as indicated in the legends), and the assay was started by the addition of creatine phosphate (5.0 mM final concentration) + MgATP in the appropriate concentration. Each ATP mixture contained in addition KCl to bring all assays to the same ionic strength (see legends). It is important to avoid mixing AMPPNP and the complete phosphorylating system during temperature equilibration of the enzyme because of the contaminating ADP + ATP in the AMPPNP.

Titration of Acto-S-1 with AMPPNP. Dissociation of acto-S-1 by AMPPNP was measured by separating the actin-bound from the free S-1 by a brief high-speed centrifugation. [^{3}H]NEM-labeled actin and native S-1 were mixed in the appropriate proportions with 5 mM $MgCl_2$ in excess over MgAMPPNP concentration at pH 7.0 (10 mM imidazole buffer) and the appropriate concentration of KCl to maintain the same ionic strength for all concentrations of MgAMPPNP. Denatured S-1, defined as that which remained in the supernatant after centrifugation with 10 μM actin in the absence of MgAMPPNP, was subtracted from each value. In the experiments with [^{3}H]NEM-labeled S-1, native actin was used, and dissociated S-1 was determined by measuring the radioactivity in the supernatants. In the experiments where native S-1 was used, dissociated S-1 was determined by the Lowry reaction (Lowry et al., 1951). Nonpolymerized [^{3}H]NEM-labeled actin, present in the supernatant, was subtracted from the Lowry protein values. A detailed protocol, control experiments, and calculations of the data have been described (Murray et al., 1981).

Modelling of the ATPase Data. The ATPase activity of S-1 in the presence of tropomyosin-free actin is satisfactorily accounted for under steady-state conditions by the Taylor–Tonomura–Trentham reaction scheme [cf. Trentham et al.

Table I: Reactions Included in the Model of Steady-State ATPase Activity of Tropomyosin Actin S-1[a]

reactants ⟷ products	$k_{forward}$	ref	$k_{reverse}$	ref
S + T ⟷ ST	5×10^6	*d, e*	1×10^{-4}	*f, g*
S + N ⟷ SN	1.0×10^6 (1.8×10^6)	*d*	$0.5\ (K_D/k_+)$	*h*
ST ⟷ ST*	70	*i, e*	30	*i, e*
ST* ⟷ S + ADP + P_i	0.1	*d*	0	*f*

reactants ⟷ products	nonpotentiated				potentiated	
	$k_{forward}$	ref	$k_{reverse}$	ref	$k_{forward}$	$k_{reverse}$
A + S ⟷ AS	5×10^6	*j*	0.1	*j, k*	5×10^7	0.1
A + ST ⟷ AST	5×10^5	*i*	1×10^3	*l*	1.5×10^6 (2.5×10^6)	1×10^3
A + ST* ⟷ AST*	3×10^5 [b]		150[b]		1.5×10^6	150
A + SN ⟷ ASN	8×10^4 [c] (7×10^5)	*c*	2		1×10^5 (7×10^5)	2 (10)
AS + T ⟷ AST	5×10^6	*l*	10	*i*	1.7×10^6	10
AS + N ⟷ ASN	3×10^5 [c] (2×10^5)		200		4×10^4	200
AST* ⟷ AS + ADP + P_i	150	*m*	0		300	0

[a] Calculations for curves 1 and 2 of Figure 4A,C and those for Figure 4B were carried out with these constants. Curve 3 of Figure 4C was obtained when some of the rate constants were changed, the values of the altered constants appearing within parentheses. For first-order reactions, the rate constants have the units s^{-1}. Second-order rate constants are given as $M^{-1}\ s^{-1}$. Several sequences of first-order steps have been compressed (e.g., those connecting to S-1) into a single step each, with a rate constant approximating the slowest step in the sequence. A = actin, N = AMPPNP, S = S-1, T = ATP, ST* = S-1-ADP·P whose binding to actin accelerates release of ADP and P_i. [b] Made to fit V_{max} and K_M for actin of steady-state ATPase. [c] k_+/k_- fitted to K_A of Greene & Eisenberg (1978) (Murray et al., 1981). [d] Bagshaw & Trentham (1974). [e] Johnson & Taylor (1978). [f] Mannherz et al. (1974). [g] Wolcott & Boyer (1974). [h] Yount et al. (1971). [i] Sleep & Hutton (1978). [j] White & Taylor (1976). [k] Marston & Weber (1975). [l] Lymn & Taylor (1971). [m] Bremel et al. (1972).

(1976), Taylor (1979), and Eisenberg & Greene (1980)]. If troponin–tropomyosin is present, the scheme must be modified to include two (in the presence of Ca^{2+}) kinetically distinguishable states of actin. For our present purpose, it must also be extended to allow for the competition between ATP and AMPPNP for the nucleotide site on S-1. Fortunately, many of the transient intermediates which have been detected influence only the non-steady-state behavior of this system. These need not be included in a model such as ours which is designed for approximate quantitative or qualitative comparison with experiments carried out entirely at steady state. The reactions included in the model and the rate constants used for the calculations of Figure 7 are listed in Table I. The values listed are the experimentally observed apparent rate constants for the reactions as written, some of which are in fact composites of two or more elementary steps. Whenever necessary, the values reported in the literature have been adjusted to make them both appropriate to our experimental conditions and thermodynamically self-consistent. The only values available are those for pure actin, corresponding to the nonpotentiated state of regulated actin. Conversion of actin from the nonpotentiated to the potentiated state alters the rate constants, as shown (Bremel et al., 1972). Calculations were carried out for a range of values of those rate constants which are not tightly constrained by kinetic studies reported in the literature, with no qualitative change in the final computed curves.

The calculation of the steady-state concentration of the various species was carried out by the method of King & Altman (1956), using a slightly modified form of the computer algorithm described by Fisher & Schulz (1969). As more than one species is conserved, the calculation of steady-state concentrations must be done in an iterative manner. (The calculation is equivalent to solving a set of nonlinear simultaneous equations.) After an initial guess at the concentration of free actin and free ATP was made, the following cycle was repeated: (1) Compute the steady-state concentration of all species corresponding to this free concentration of actin and ATP. Form the appropriate sums to compute total actin and total ATP concentrations. (2) In general, the computed totals will differ from those used in the actual experiment being modelled. Adjust the free concentrations of ATP and actin so as to bring the total concentrations closer to their desired values. (3) Calculate the distribution of actin between potentiated and nonpotentiated states. Adjust this distribution as necessary to match the expected distribution, determined by the procedure described later. The adjustment is made by altering the fraction of the total free actin apportioned to the free potentiated and to the free nonpotentiated states. (4) Use the revised free concentrations of actin and ATP for the next cycle beginning at step 1. The cycle was terminated when the calculated concentrations were no longer changing significantly, and the total concentrations of actin and ATP matched the experimental values.

The expected distribution of actin between potentiated and nonpotentiated states is modelled by making the following three postulates: (1) The transition between potentiated and nonpotentiated states of actin occurs simultaneously for all of the seven actin monomers covered by a single tropomyosin. (2) A group of seven is promoted to the potentiated state whenever it collectively binds two or more S-1 molecules. (3) Among actins in the same state, the distribution of bound S-1's was assumed random. The fraction of seven-membered groups of nonpotentiated actin monomers containing at least two bound S-1 molecules can then be calculated from the concentrations of the various species computed in step 1 of the cycle above (i.e., as a multinomial distribution). This fraction of the total nonpotentiated actin was transferred to the potentiated state. Similarly, those groups of potentiated actin containing less than two bound S-1's were transferred to the nonpotentiated state. After a few iterations of the cycle, the net transfer between states became insignificant; this was one of the conditions for terminating the calculation.

Results

Figures 1 and 2 describe potentiation of the ATPase activity of regulated acto-S-1. Since we have observed previously (Murray et al., 1980a,b) that calcium-saturated troponin does not significantly alter the effects of tropomyosin on the cofactor activity of actin, we chose for the sake of convenience to use troponin–tropomyosin actin in the presence of calcium rather than tropomyosin actin. Figure 1A shows, first, the enhancement of acto-S-1 ATPase activity by calcium-saturated troponin–tropomyosin which was mostly due to an increase in the V_{max} (Figure 1B). Second, Figure 1A shows that this enhancement of acto-S-1 ATPase activity depended on S-1 saturation of the actin filament in a cooperative manner as indicated by the typically sigmoid shape of the curve (Hill coefficient of about 1.7). This contrasts with the ATPase activity of tropomyosin-free acto-S-1 which increased with

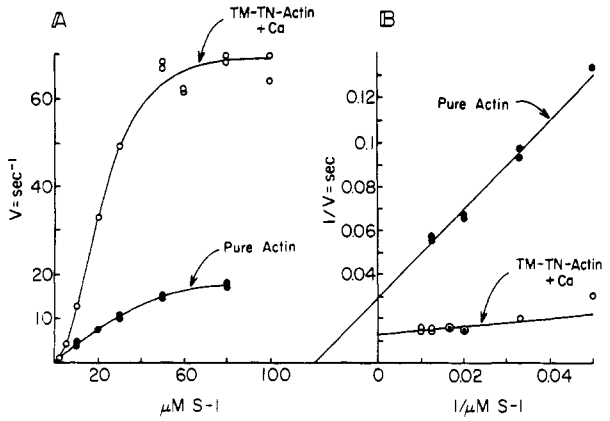

FIGURE 1: Potentiation of acto-S-1 ATPase activity (in the presence of troponin and tropomyosin) with increasing S-1 concentrations compared to the noncooperative behavior of pure acto-S-1. TM–TN–actin represents tropomyosin–troponin actin; the following conditions existed: 1.5 μM actin, 25 °C, 0.2 mM $CaCl_2$, 1.0 mM CaEGTA, 1.0 mM MgATP. ATP hydrolyzed by S-1 alone was subtracted from the total ATP hydrolyzed. (A) Raw data; (B) double-reciprocal plots of both acto-S-1 preparations; however, for TM–TN–acto-S-1, only the highest data points were plotted for extrapolation to the K_M(S-1) of the final potentiated state. Values for V_{max} were 33 and 75–100 mol of ATP hydrolyzed·(mol of actin)$^{-1}$·s^{-1} for pure and fully potentiated regulated actin, respectively.

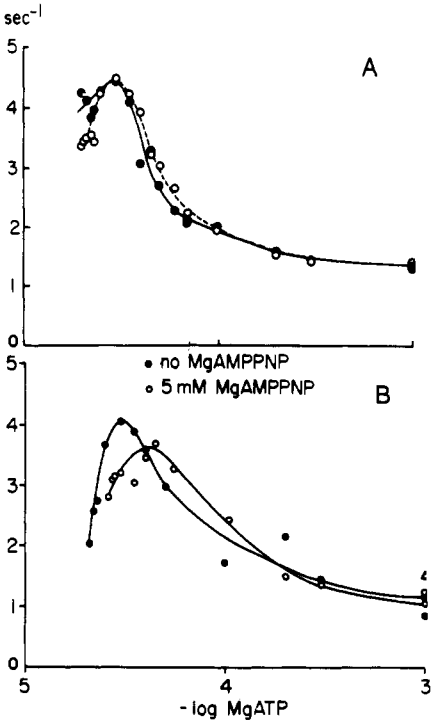

FIGURE 2: Potentiation of ATPase activity at low MgATP concentrations with and without MgAMPPNP. Two experiments with different protein preparations representative of the variations in results that were encountered. Troponin–tropomyosin actin (7 μM) and 20 μM S-1 were present. (○) AMPPNP present; (●) controls. Abscissa = total MgATP concentration; ordinate = moles of creatine liberated per second per mole of actin. ATP hydrolyzed by S-1 without actin and in the absence of AMPPNP was substracted from all values, including the values of acto-S-1 ATPase activity in the presence of AMPPNP. Modelling of the experiments shows that in the presence of actin the fraction of the free S-1 containing AMPPNP is smaller than that in the absence of actin. If we had subtracted ATP split by S-1 alone in the presence of AMPPNP, the rates of hydrolysis by acto-S-1 in the presence of AMPPNP would have been 3–10% higher. This experiment had the following conditions: 5 mM MgAMPPNP and 5 mM $MgCl_2$, in excess MgAMPPNP; ionic strength 60 mM; 0.2 mM $CaCl_2$ and 1.0 mM EGTA; 25 °C. The ATP + ADP present in the AMPPNP (5 μM) was corrected for. The rates of ATP hydrolysis are low because of the summation of the depressing effects of high ionic strength and high Mg^{2+} concentrations. Panels A and B show small differences in the kinetics of potentiation in the presence of AMPPNP. The open triangle is the rate of ATP hydrolysis when the troponin–tropomyosin complex had been omitted.

increasing S-1 concentrations according to Michaelis–Menten kinetics (Eisenberg & Moos, 1970). In other words, whereas in the case of pure acto-S-1 none of the rate constants that determine ATP turnover were affected by increasing S-1 concentrations, some or all of these rate constants changed with changing S-1 concentrations when calcium-saturated troponin–tropomyosin was present. In the double-reciprocal plot of Figure 1B, we extrapolated five points near the plateau of the troponin–tropomyosin acto-S-1 curve to the abscissa intercept. Although one cannot obtain an accurate number for the abscissa intercept from the data of Figure 1 (or our other experiments) because it is too difficult to decide at which point the rate constants have reached their final value, the abscissa intercept for troponin–tropomyosin acto-S-1 seems to be at a somewhat lower S-1 concentration than the abscissa intercept for pure acto-S-1. The rate constants that determine the abscissa intercept represent the K_M for S-1 when the tropomyosin actin filament was in the fully potentiated state, i.e., that K_M should not be identical with the S-1 concentration at the apparent midpoint of the titration curve. Figure 1 seems to suggest that like nucleotide-free acto-S-1 complexes (rigor complexes) (Bremel et al., 1972), nucleotide-containing acto-S-1 complexes also can cause potentiation since 1.0 mM ATP concentrations are presumably saturating. This conclusion had been reached earlier by Bremel and Murray (Bremel et al., 1972) on the basis of a similar experiment. Meanwhile, however, we learned that rigor complexes can be formed at saturating ATP concentrations by partially denatured S-1 molecules whose actin binding site is still intact while their ATP site is damaged (Pemrick & Weber, 1976). Such S-1 is no longer dissociated from actin by ATP and thus forms "ATP resistant rigor complexes" (Pemrick & Weber, 1976). Since the S-1/actin ratio at which potentiation occurred in Figure 1 was rather large, even a relatively small contamination with such damaged S-1 could have provided enough rigor complexes to cause potentiation.

Potentiation of Acto-S-1 ATPase Activity by MgAMPPNP. In order to determine the capacity of nucleotide S-1–actin complexes to cause potentiation, we used the ATP analogue 5′-adenylyl imidodiphosphate (AMPPNP). S-1–AMPPNP can be used at low enough concentrations to avoid interference by a small contamination with partially denatured S-1 capable of forming ATP-resistant rigor complexes.

The experiment of Figure 2 shows that acto-S-1–AMPPNP complexes can cause potentiation. Two typical responses found with different proteins at different times are described by Figure 2A,B. The control curves in the absence of AMPPNP show potentiation by rigor complexes as first described by Bremel and colleagues (Bremel et al., 1972). In this experiment, both actin and S-1 concentrations were kept constant (S-1/actin = 3), and the ATP concentration was gradually raised to saturating levels, thereby gradually reducing the steady-state concentration of rigor complexes. ATP hydrolysis was increased with increasing ATP concentration in proportion to the increasing liberation of free actin from rigor complexes as long as the concentration of rigor complexes remained at or above the level necessary for the maintenance of the potentiated state. On further reduction of the concentration of rigor complexes, the rate of ATP hydrolysis gradually returned to a lower rate comparable to that of pure acto-S-1. The potentiated rate of ATP hydrolysis reached a maximum at about 10–20 μM ATP in excess over S-1[2] when it was about

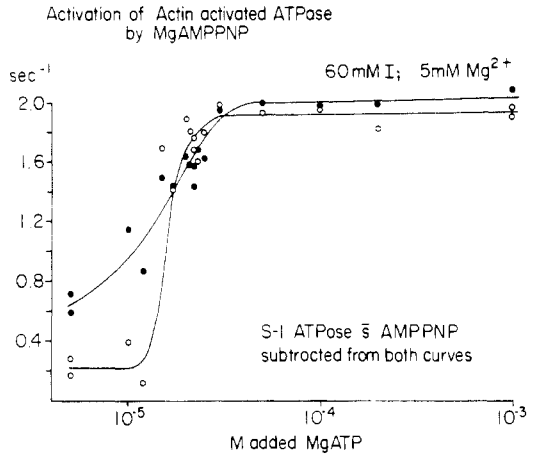

FIGURE 3: Control experiment for Figure 2: the effect of AMPPNP on acto-S-1 ATPase activity with pure actin with increasing ATP concentrations. Pure actin (3 μM) and 20 μM S-1 were present; all other conditions are as described in Figure 2.

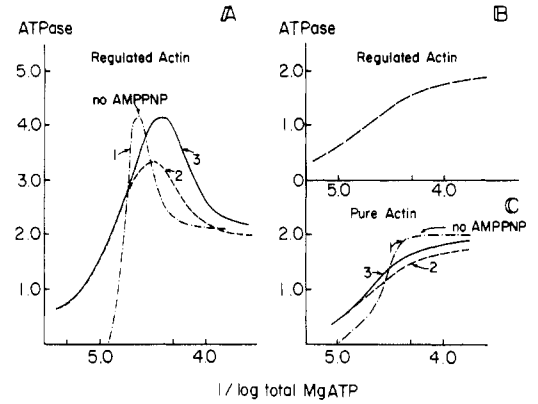

FIGURE 4: Modelled curves of the effect of AMPPNP on acto-S-1 ATPase activity. (A and B) Regulated actin; (C) pure actin. (A) Acto-S-1–AMPPNP complexes are as capable as rigor complexes to cause potentiation. (B) Acto-S-1–AMPPNP complexes cannot cause potentiation. The rate and binding constants are enumerated in Table I. The curves in (A) and (C) differ with respect to the binding constants of AMPPNP to acto-S-1 and of S-1–AMPPNP to actin, and in (A) with respect to the response to potentiation of the binding constant of AMPPNP to acto-S-1.

Table II: Beginning Transition from the Nonpotentiated to the Potentiated State[a]

S-1/actin ratio	acto-S-1 ATPase $V_{pure}/V_{regulated}$ actin ratio
0.017	1.74
0.033	1.33
0.125	0.89

[a] 0.15 μM S-1, 10–1.2 μM actin (either pure or reconstituted with the tropomyosin-troponin complex), and 1.0 mM MgATP were present; other conditions are as described for Figure 1. The nonpotentiated state is indicated by a high $V_{pure}/V_{regulated}$ (=Ca^{2+}-saturated troponin-tropomyosin actin) actin ratio. Increasing the S-1/actin ratio initiates the transition toward the potentiated state.

3-fold higher than the final plateau rate of the same acto-S-1 free of tropomyosin (single triangle at 1.0 mM MgATP[3]). The minimum level of rigor complexes associated with ATP saturation was reached at ATP concentrations above 100 μM. That is a much higher concentration of ATP than that necessary to saturate pure acto-S-1. The control curve of Figure 3 shows that the rate of hydrolysis of pure acto-S-1 reached its final plateau value when ATP concentration became stoichiometric (or very slightly in excess of stoichiometric) to S-1 concentration, indicating that the K_M for ATP is lower for pure than for regulated acto-S-1 under potentiating conditions as previously observed (Bremel et al., 1972; Murray et al., 1980b). The biphasic response of ATP hydrolysis persisted on addition of 5 mM MgAMPPNP; MgAMPPNP did not significantly depress the rate of ATP hydrolysis in any range of ATP concentrations (Figure 2). Apparently AMPPNP was not an effective competitive inhibitor either in the absence (Figure 3) or in the presence of troponin–tropomyosin, although it competes well with ATP when S-1 hydrolyzes ATP in the absence of actin (Yount, 1975; Bagshaw et al., 1972). Only in the range of the transition from substoichiometric to stoichiometric ATP concentration was there a very slight competitive inhibition by AMPPNP both with pure and with regulated acto-S-1.

However, AMPPNP binds to rigor complexes in the absence (Greene & Eisenberg, 1978; Murray et al., 1980a, 1981) and in the presence of troponin–tropomyosin (see below), setting up an equilibrium between acto-S-1–AMPPNP complexes and actin + S-1–AMPPNP. As a result, AMPPNP *activated* ATPase activity in the control experiment with *pure* acto-S-1 (Figure 3) when the ATP concentration was substoichiometric to that of S-1, and therefore, hydrolysis rates in the absence of AMPPNP were limited by the availability of free actin necessary for the release of ADP and phosphate from S-1–ADP·P. (To simplify data analysis, we avoided this concentration range in the experiments with regulated acto-S-1.) When we calculated the approximate steady-state concentrations of rigor complexes, acto-S-1–AMPPNP, free actin, S-1–AMPPNP, and the intermediates of acto-S-1–ADP·P formation (see Materials and Methods and Table I), we found that 5 mM MgAMPPNP reduced the steady-state concentration of rigor complexes to levels insufficient to maintain the potentiated state without support from the acto-S-1–AMPPNP complexes. That is indicated by Figure 4B showing rates of ATP hydrolysis modelled for the condition that acto-S-1–AMPPNP is incapable of inducing potentiation of ATPase activity: the biphasic response is lost, and hydrolysis increases monotonically with increasing ATP concentration, comparable to the ATPase activity of pure acto-S-1 or regulated acto-S-1 with an actin/S-1 ratio of 100 (Bremel et al., 1972). Modelling showed that the biphasic response was retained only if acto-S-1–AMPPNP complexes were capable of causing potentiation (Figure 4A).

S-1 Binding to Potentiated and Nonpotentiated Actin Filaments. The potentiation of ATPase activity has been attributed by us to a change in the actin filament from a nonpotentiated to a potentiated state (Bremel et al., 1972; Weber & Murray, 1973; Murray et al., 1980b). In the framework of this hypothesis, potentiated actin filaments are much better cofactors of ATPase activity than regulated nonpotentiated or pure actin filaments. We attempted to further characterize these states of the actin filament by comparing their binding constants for S-1 and for S-1–AMPPNP. Since we were primarily interested in the differences between the states and not in the transition states, the experiments were designed to avoid any such transitions during our measurements. Earlier experiments (Bremel et al., 1972) suggest that regulated actin filaments are in the nonpotentiated state when the actin/S-1

[2] That this ATP concentration is lower than in previous experiments (Bremel et al., 1972; Murray et al., 1975, 1980a,b) is related to the rather high ionic strength and the high concentration of free Mg^{2+}.

[3] ATPase activity of tropomyosin-free S-1 always reaches plateau values with increasing ATP concentration; i.e., rates of ATP hydrolysis at concentrations below 1.0 mM are the same or lower than those at 1.0 mM ATP.

Table III: Changes in Association Constants for S-1–AMPPNP Binding to Actin (K_1) and for AMPPNP Binding to Acto-S-1 (K_2) When Tropomyosin-Free Actin (Pure A) Is Substituted by Troponin–Tropomyosin Actin (RA) under Nonpotentiating Conditions in the Presence of Ca^{2+} [a]

	protein concn (μM)			intercepts		intercept ratios
		actin				
expt	S-1	pure A	RA	ordinate, K_1 ($\times 10^5$ M^{-1})	abscissa, K_2 ($\times 10^3$ M^{-1})	RA/PA = K_3(RA)/K_3(PA) [b]
1 [c]	10		14	1.0	1.0	6
	0.66		21	0.67	3.0	1.4 [f]
	10	14		0.73	4.5	
2	14		18	0.5	0.5	3.4
	14	18		0.5	1.7	
3	14		17	0.7	0.4	4.8 [e]
	1		17	0.3	0.83	
4 [d]	10		15	0.7	0.25	5
	10	15		0.7	1.25	
5	6.5		9.5	0.7	0.4	5
	6.5	9.5		0.7	2	

[a] 0.1 mM $CaCl_2$ was present in all assays. [b] [Ordinate/abscissa (RA)]/[ordinate/abscissa (PA)]. [c] Experiment of Figure 6. [d] Experiment of Figure 5. [e] Intercept ratios between high and low S-1/actin ratio; both actins = RA. [f] Note that under nonpotentiating conditions, the intercept ratio between RA and PA is close to 1. K_1 = [AS-AMPPNP]/([A][S-1-AMPPNP]), K_2 = [AS-AMPPNP]/([AS][AMPPNP]), and K_3 = [AS]/([A][S-1]) whereby AS = acto-S-1 and A = actin. The constants of experiments 4 and 5 were obtained by directly fitting the raw data (equal to the percent S-1 dissociated). They are within a factor of 2 and 10% of the constants obtained from the weighted double-reciprocal plot for experiments 4 and 5, respectively.

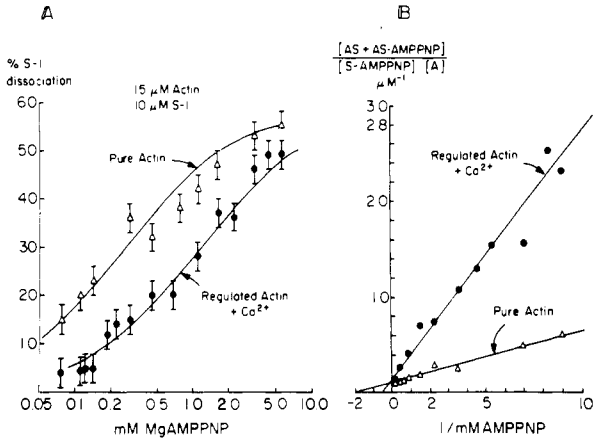

FIGURE 5: S-1 binding to actin, comparing troponin–tropomyosin actin under potentiating conditions with tropomyosin-free actin. Native S-1 and actin labeled at Cys-373 with *N*-[^{3}H]ethylmaleimide; 5 mM $MgCl_2$ in excess over MgAMPPNP concentration, 0.2 mM $CaCl_2$, 1.0 mM CaEGTA, 60 mM ionic strength, 15 °C. The error bars in (A) indicate the maximal uncertainty (=6%) in the fraction of denatured S-1. The lines through the data of (A) are the theoretical lines for the constants given in Table III (experiment 4).

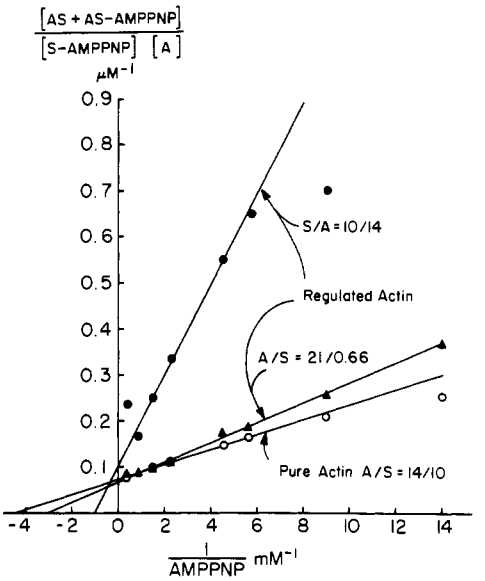

FIGURE 6: S-1 binding to actin as a function of increasing AMPPNP concentrations, comparing pure actin with troponin–tropomyosin actin under nonpotentiating (low S-1/actin ratio) and potentiating conditions (high S-1/actin ratio). All other conditions are as described in Figure 5. Native actin = *N*-[^{3}H]ethylmaleimide-labeled S-1; pure actin = tropomyosin-free actin; regulated actin = troponin–tropomyosin actin; the numbers present by the A/S (actin/S-1) ratios indicate the concentration of each protein in micromolar. Values for intercepts are given in Table III.

ratio is greater than 20 (Table II) and that they are in the fully potentiated state when the saturation of the regulated actin filament with S-1 equals or exceeds 30%. For instance, for the experiment of Figure 5, the potentiated regulated actin started with an S-1 saturation of 67% which did not fall below 35% at the highest AMPPNP concentration used. We measured the dissociation of acto-S-1 by increasing concentrations of MgAMPPNP. Figure 5A shows directly that more MgAMPPNP is needed to dissociate potentiated regulated acto-S-1 than pure acto-S-1. The reciprocal plot of Figure 6 shows that dissociation of pure acto-S-1 by AMPPNP is similar to that of nonpotentiated regulated acto-S-1. The dissociation of S-1 from pure actin was only affected very little by the degree of saturation with S-1 (panels A and B of Figure 7 represent two different experiments, one with a change in the ordinate intercept and the other in the abscissa intercept, respectively). Furthermore, the small change was in the opposite direction from that with regulated actin: in three out of three experiments, S-1 binding to actin seemed to be stronger when the S-1/actin ratio was low (see below for analysis of the data).

These data can be analyzed (Greene & Eisenberg, 1978; Murray et al., 1981) by plotting the ratio of bound S-1 concentration to the product of the concentrations of free S-1 and free actin vs. the reciprocal of the AMPPNP concentrations. The linearity of the reciprocal plots suggests that none of the relevant binding constants was altered (over the range of AMPPNP concentrations measured) by cooperative interactions due to changing S-1 saturation of the actin filaments. The binding constant of S-1–AMPPNP for actin is given by the ordinate intercept. This binding constant was not significantly different for tropomyosin-free actin and for troponin–tropomyosin actin in the potentiated state (Table III).

The abscissa intercept gives the apparent binding constant for AMPPNP to actin-bound S-1. It was 2–4 times higher for S-1 bound to potentiated troponin–tropomyosin actin than

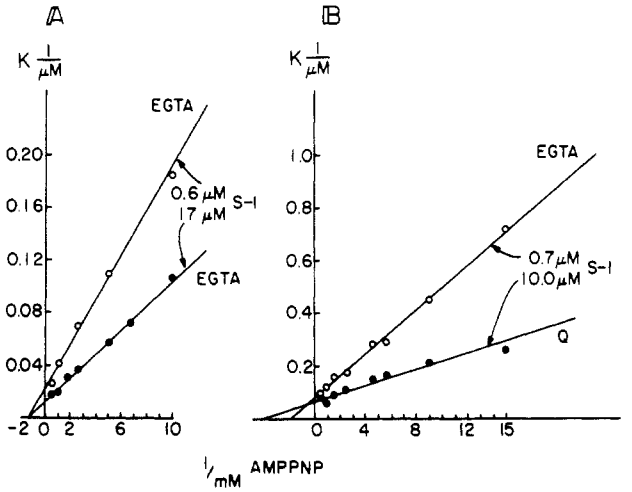

FIGURE 7: Effect of raising the S-1/actin ratio on S-1 binding to tropomyosin-free actin. Conditions are as described in Figure 5. S-1 concentration is as indicated on curves; (A) 20 μM actin; (B) 14 μM actin.

for S-1 bound to tropomyosin-free actin (Table III). The ratio of the ordinate to the abscissa intercept give the ratio of the apparent rigor complex association constant to the apparent binding constant of AMPPNP to free S-1 (Murray et al., 1981). Thus, the apparent association constant for rigor complex formation can be calculated if the binding constant for AMPPNP to free S-1 is known (Bagshaw et al., 1972). Without the value for the apparent binding constant of AMPPNP to free S-1, one cannot obtain an absolute value for the rigor binding constant. However, since AMPPNP binding to free S-1 is unaffected by the state of the actin filaments (Murray et al., 1981), the *change* in the apparent rigor complex association constant associated with a change in the state of the actin filaments can be calculated from the *change* in the intercept ratio. Rigor complex formation was 3–6 times stronger with potentiated than with pure actin. Since the values for pure actin were similar to those for nonpotentiated troponin–tropomyosin actin (Figure 6 and Table III), the transition from the nonpotentiated to the potentiated state resulted in a 3–6-fold increase in the affinity of actin for nucleotide-free S-1.

Discussion

Comments about Individual Experiments. Potentiation changed two parameters of acto-S-1 ATPase activity: the V_{max} was increased and the K_M for S-1 slightly decreased. The increase in V_{max} has not been reported before but was observed now in five out of six experiments. It is possible that the V_{max} change was missed in earlier experiments: the extrapolations were much less accurate then, because very high values of V_{max} and K_M for S-1 made it possible to raise the S-1 concentrations to the value of the K_M. Although it is most likely that the increase in V_{max} was always present but remained unobserved because of the uncertainties of extrapolation, one cannot be certain of that. It must be pointed out that the protein preparations used in recent years differ from those used by Bremel and by Murray (Bremel et al., 1972): the recent preparations show a lower V_{max} and K_M, more in the range of values observed by other laboratories [cf. Taylor (1979)] than the earlier ones. We do not know whether to attribute this to a change in actin or in S-1. The only systematic differences in protein preparation consist of taking up actin pellets in 2.0 mM $MgCl_2$ instead of 0.1 M KCl and 1.0 mM $MgCl_2$ and in selecting from the total fraction of papain or chymotryptic S-1 that which precipitates between 50 and 55% ammonium sulfate (see Materials and Methods).

We would like to stress that the enhancement by AMPPNP of the ATPase activity of pure acto-S-1 at substoichiometric ATP concentrations (Figure 3) was completely unrelated to potentiation. In the absence of AMPPNP, ATP hydrolysis was near zero because all or most of the actin was complexed with S-1 in rigor complexes. The partial dissociation of rigor complexes by 5 mM AMPPNP (Greene & Eisenberg, 1978; Marston et al., 1976; Yount, 1975) provided free actin to accelerate the release of ADP and inorganic phosphate from S-1–ADP·P.

One may have expected that AMPPNP in concentrations in excess of S-1 concentration would inhibit competitively acto-S-1 ATPase activity more strongly than was observed. AMPPNP binds to the ATP–ADP site of S-1 where it can be trapped like ADP (Wells & Yount, 1979), and it strongly inhibits ATPase activity of S-1 alone (Bagshaw et al., 1972; Yount et al., 1971). In the absence of actin, AMPPNP binds to S-1 with an apparent binding constant of about 10^6–10^7 M^{-1} (Bagshaw et al., 1972). The affinity of AMPPNP to actin-bound S-1 (the final intermediate of acto-S-1 ATPase activity) is reduced by several orders of magnitude to 2×10^3 M^{-1} for pure actin (Green & Eisenberg, 1978; Murray et al., 1981) and to an even lower value (5×10^2 M^{-1}) for troponin–tropomyosin actin in the potentiated state (Figure 5, Table III). Thus, in the presence of actin, there is much less free S-1–AMPPNP at a given ATP/AMPPNP ratio than in its absence. Furthermore, for each concentration of total ATP in Figures 2 and 3, the concentration of free ATP was greater in the presence of AMPPNP than in its absence. In addition, at ATP concentrations near stoichiometric to S-1 concentration, inhibition was counteracted by activation due to actin release from rigor complexes as just discussed. However, we also did not observe significant competitive inhibition under conditions of actin excess and S-1 concentrations in the range of 0.1–0.15 μM (not shown here) where inhibition would not be obscured by the last two factors. Since contamination of ADP + ATP limits the AMPPNP/ATP ratio to 1000/1, 2 mM AMPPNP would be expected to cause 50% inhibition at 2 μM ATP (corrected for the contaminating ATP + ADP) unless the K_M for ATP was more than 3 orders of magnitude lower than the K_D of AMPPNP for actin-bound S-1. The lack of inhibition under these conditions suggests that the K_M for ATP was below 0.5 μM when free actin concentration was in the range of 2–5 μM.[4]

The points made in the above discussion were reasonably well verified by modelling of the data. The modelled curves 1 and 3 of Figure 4C (pure acto-S-1) are comparable with Figure 3 although competitive inhibition is somewhat more marked in Figure 4C, and the modelled competitive inhibition of Figure 4A (potentiated acto-S-1) is comparable to the observed inhibition of Figure 2B. A comparison of the two curves in Figure 4A,C shows how sensitively the shape of the curves depends on the values of the binding constants of AMPPNP to acto-S-1 and possibly of S-1–AMPPNP to actin.

[4] Such a low K_M is lower than that suggested by direct measurements of this K_M using a rephosphorylating system to maintain a constant ATP level at low ATP concentrations (Bremel et al., 1972; Murray, 1973). However, discrepancies between a K_M for ATP measured with a phosphorylating system and calculated from other data, such as rate constants, have been observed before (Taylor & Weeds, 1976). We suspect that in these cases the steady-state concentration of ADP and of ATP bound to the phosphorylating enzymes (which must be present in relatively high concentration to compensate for their relatively high K_M values for ADP) is a significant fraction of the total ATP concentration.

The curves of Figure 4A, modelled with the assumption that acto-S-1–AMPPNP complexes are indistinguishable from acto-S-1 complexes in their ability to cause potentiation, reproduce qualitatively all of the features of the experimental curves of Figure 2. (The enhancement—unrelated to potentiation—of ATPase activity of ATP concentrations substoichiometric to S-1 concentration discussed above is not seen in Figure 2 because we did not go to such low ATP concentrations.) Both the modelled and the experimental curves (1) show biphasic behavior in the presence of AMPPNP, (2) show a slight depression of ATPase activity in the presence of AMPPNP at ATP concentrations slightly in excess of S-1 concentration, probably due to competitive inhibition between ATP and AMPPNP, (3) show potentiation in the presence of AMPPNP to drop off at slightly higher ATP concentrations than in the presence of AMPPNP, and (4) comparing only Figure 2B, show AMPPNP to shift the potentiation peak to slightly higher ATP concentrations. The difference between panels A and B of Figure 2 is presumably due to differences in the rate and binding constants between the two sets of protein preparations.

We do not have enough data to conclude from the experiment of Figure 7 that there is negative cooperativity of S-1 binding to pure actin. If these data should be confirmed, they might be related to the conformational changes in pure actin filaments observed by Thomas and his colleagues (Thomas et al., 1979).

Relationship between S-1 Binding to Actin and Acto-S-1 ATPase Activity. The potentiation of ATPase activity is due to a potentiation of the cofactor activity of actin, i.e., a change in the state of the regulated actin filament. The transition from the nonpotentiated to the potentiated state was caused by an increase in the saturation of the regulated actin filament either with S-1 or, as shown here, with S-1–AMPPNP. Therefore, one expects that S-1 or S-1–AMPPNP binds more strongly to the potentiated than to the nonpotentiated actin filament (Monod et al., 1965). The present data show such a difference in the binding of S-1.

However, the apparent binding constant of S-1–AMPPNP to regulated nonpotentiated and to potentiated actin appeared to be the same. That is not inconsistent if one takes into account the fact that the transition responsible for the change of state is a transition made presumably by a complex of one tropomyosin and seven actin molecules. When we measured the binding of S-1–AMPPNP to nonpotentiated actin, we measured binding to, on the average, much less than one out of seven actin molecules. It is possible to assume that there would be negative cooperativity of binding of S-1–AMPPNP to regulated nonpotentiated actin filaments so that the binding constant for two S-1's and seven actins was much lower than that for one S-1 and seven actins, as indicated by Figure 8. In that case, increased saturation of the actin filament with S-1–AMPPNP would shift the equilibrium of the complex to the potentiated state. Comparing the energy squares with S-1 and with S-1–AMPPNP (Figure 8), one finds that the degree of saturation necessary to shift the equilibrium 90% toward the potentiated state can be made the same with S-1 and S-1–AMPPNP so as to fit our modelling of the data of Figure 2. However, it cannot be avoided that in the case of negative cooperativity the transition is sharper than that for S-1 unless S-1 also has negative cooperativity of binding to the nonpotentiated state.

In conclusion, the observed increase in the apparent actin binding constant of S-1 associated with the shift to the potentiated state is consistent with the observation that rigor

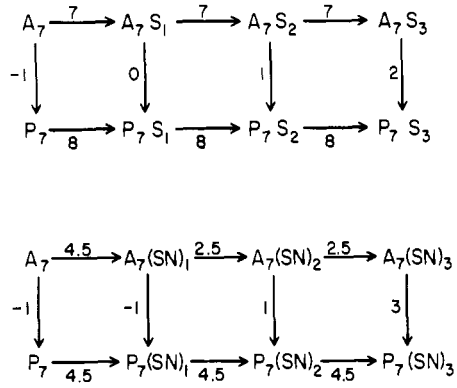

FIGURE 8: Energy squares describing the transition from the nonpotentiated to the potentiated state caused by either S-1 or S-1–AMPPNP. A_7 and P_7 represent a unit of seven actin monomers (associated with one tropomyosin molecule) either nonpotentiated (A) or potentiated (P); S represents S-1; (SN) represents S-1–AMPPNP. The numbers on the arrows are the log of the equilibrium constants, positive if the equilibrium is in the direction of the arrow. With increasing saturation with either S-1 or S-1–AMPPNP, the equilibrium is shifted toward the potentiated state.

complexes cause potentiation of ATPase activity. However, it is not obligatory that, as measured, the first out of seven S-1 molecules should have been bound more weakly in the nonpotentiated than in the potentiated state. Thermodynamics would have been satisfied if negative cooperativity of binding to nonpotentiated filaments had supplied the difference in binding energy between the two states, as it apparently did in the case of S-1–AMPPNP binding to actin. Green & Eisenberg (1980) observed an increase in the binding of S-1–ADP when the actin filament changed from the "weak" to the "strong" state. The transitions from the weak to strong state and from the nonpotentiated to the potentiated state are caused by about the same increase in S-1 saturation of the regulated actin filaments, suggesting that both nomenclatures describe the same states. Quantitatively their observed increase in binding of S-1–ADP, described by the experimental term [bound S-1–ADP]/([free actin][free S-1–ADP]), is much larger (by a factor of more than 10) than the difference we determined for S-1 binding. However, the very low S-1–ADP binding in Table I of the paper by Greene & Eisenberg (1980) when the S-1–ADP/actin ratio was 0.01–0.02 is not consistent with their value for $L = 7$ that was used to model the rest of the binding curve, a point not commented on in the paper. We predict that S-1–ADP also causes potentiation of ATPase activity so that the results would be qualitatively similar if in the experiment of Figure 2 ADP had replaced AMPPNP.

The ATPase activities of pure actin and regulated actin in the nonpotentiated state are different; i.e., the presence of the regulatory proteins (or tropomyosin alone) inhibits ATPase activity by about 60% (Bremel et al., 1972; Murray et al., 1975, 1980b; Eaton et al., 1975).

In this case, one cannot predict what the relationship between ATPase activity and S-1 binding should be. Greene & Eisenberg (1980) observed that less S-1–ADP was bound to regulated actin than to pure actin when the S-1/actin ratio was below 0.1 whereas we did not observe any significant difference in S-1 binding under similar conditions. The correlation between S-1 binding and ATPase activity is of interest from the point of view of the mechanism of acto-S-1 ATPase. Thus, studying relaxation by removal of calcium, Chalovich and his colleagues (Chalovich et al., 1981) and Wagner & Giniger (1981) recently observed that binding of ATP-saturated S-1 to relaxed actin filaments apparently was not inhibited under conditions where ATPase activity has been

20-fold reduced. Inhibition of ATPase activity during relaxation seemed to be strongly correlated to the inhibition of forming an acto-S-1–ADP complex (Greene & Eisenberg, 1980) and an acto-S-1 complex (Murray et al., 1981). In spite of our data, one cannot firmly conclude that the inhibition of ATPase activity by troponin–tropomyosin in the presence of calcium is not paralleled by any inhibition of S-1 binding for the following reason. We have recently observed (unpublished data) that not all preparations of troponin–tropomyosin or all preparations of tropomyosin cause inhibition of ATPase activity even at S-1/actin ratios as low as 0.01. Furthermore, occasionally a preparation that caused inhibition when freshly prepared lost that capacity with time. That phenomenon is under investigation now. At the time of the binding studies, we were not yet aware of this phenomenon and therefore limited our quality checks of the regulatory proteins to their ability to cause relaxation and did not check the ability to inhibit ATPase in the presence of calcium at low S-1/actin ratios. Whether or not there is a correlation between inhibition of S-1 binding and ATPase activity in the nonpotentiated state of regulated actin must be settled in the future.

How the Data Fit the Steric Blocking Model of Tropomyosin Action. Rather than discussing the different states of the actin filament in terms of conformational changes with unknown structural basis, we try to describe them, if possible, in terms of the elegant steric blocking model of tropomyosin action (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973). Thus, we have explained the inhibition of ATPase activity by calcium-saturated troponin–tropomyosin, i.e., in the nonpotentiated state, by steric blocking of a large fraction of the actin sites necessary for actin activation of ATP turnover (Murray et al., 1980a,b). Since the binding constant of a single tropomyosin molecule to seven actin monomers is very low (Wegner, 1979), we assume that tropomyosin does not bind to a specific site on actin. We consider it likely that tropomyosin binds nearly equally well to different parts of the actin surface so that the different segments of the tropomyosin strand are combined with different actin sites. Thus, some but not all of the S-1 binding sites are blocked by tropomyosin, which is consistent with the observation that the inhibition of ATPase by tropomyosin is entirely due to an increase in the apparent K_M for actin. This model which was made to fit the ATPase data is compatible with the data on binding of S-1–ADP to nonpotentiated actin filaments (Greene & Eisenberg, 1980), assuming that S-1–ADP binding is blocked in actin molecules unable to activate ATP turnover. Our model differs from the model underlying the analysis of the data by Greene and Eisenberg (Hill et al., 1980), insofar as they assign to the accessible actin molecules the constants of the potentiated ("strong" in their nomenclature) actin and not of pure actin. We consider the observation that the V_{max} of nonpotentiated ATPase activity is very close to that of pure acto-S-1 ATPase activity as an indication that the accessible actin molecules have the constants of pure actin. Our model is also compatible with the present S-1 binding data if one assumes that complete blocking of cofactor activity is associated with a decrease in the apparent actin binding constant of S-1 rather than with complete blocking of S-1 binding. In other words, S-1 but not S-1–ADP can bind to actin molecules incapable of activating ATP turnover but binds much more weakly because a major part of its binding site is blocked by tropomyosin. That assumption is supported by our recent binding studies with relaxed actin filaments which showed that usually ATPase activity was inhibited to a greater extent than S-1 binding to actin (Murray et al., 1981).

According to the model, the regulated actin filament adopts the potentiated state when tropomyosin has been displaced into the groove by the competing actin binding of S-1, S-1–ADP, or S-1–AMPPNP. However, the steric blocking model cannot explain why V_{max} and the actin binding constants of S-1–ADP and S-1 are greater for potentiated acto-S-1 than for pure acto-S-1 and why tropomyosin binds more strongly to actin filaments in the potentiated state (Eaton, 1976). That must be explained by an additional event, such as a direct interaction between groove-bound tropomyosin with S-1 or a conformational change of actin induced by tropomyosin bound to the groove sites of actin.

Acknowledgments

We thank David Trentham for a very helpful critical reading of the manuscript, and we are very grateful to E. W. Taylor for a superb refereeing of the manuscript and the suggestion to draw the theoretical curve through the data points of Figure 5A.

References

Adelstein, R. S., & Eisenberg, E. (1980) *Annu. Rev. Biochem. 49*, 921–956.

Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331–349.

Bagshaw, C. R., Eccleston, J. F., Trentham, D. R., Yates, D. W., & Goody, R. S. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 127–135.

Barany, M., & Barany, K. (1959) *Biochim. Biophys. Acta 35*, 293.

Bremel, R. D., Murray, J. M., & Weber, A. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 267–275.

Chalovich, J. M., Chock, P. B., & Eisenberg, E. (1981) *J. Biol. Chem. 256*, 575–578.

Eaton, B. L. (1976) *Science (Washington, D.C.) 192*, 1337–1339.

Eaton, B. L., Kominz, D. R., & Eisenberg, E. (1975) *Biochemistry 14*, 2718–2725.

Ebashi, S., & Ebashi, F. (1964) *J. Biochem. (Tokyo) 55*, 604–613.

Ebashi, S., Endo, M., & Ohtsuki, T. (1969) *Q. Rev. Biophys. 2*, 351–384.

Eisenberg, E., & Moos, C. (1970) *J. Biol. Chem. 245*, 2451–2456.

Eisenberg, E., & Greene, L. E. (1980) *Annu. Rev. Physiol. 42*, 293–309.

Fisher, D. D., & Schulz, A. R. (1969) *Math. Biosci. 4*, 189–200.

Greene, L. E., & Eisenberg, E. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 54–58.

Greene, L. E., & Eisenberg, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2616–2620.

Haselgrove, J. C. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 341–352.

Hill, T. L., Eisenberg, E., & Greene, L. (1980) *Proc Natl. Acad. Sci. U.S.A. 77*, 3186–3190.

Huxley, H. E. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 361–376.

Johnson, K. A., & Taylor, E. W. (1978) *Biochemistry 17*, 3432–3442.

King, E. L., & Altman, C. (1956) *J. Phys. Chem. 60*, 1375.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowey, S., Slaytor, H. S., Weeds, A. G., & Baker, A. (1969) *J. Mol. Biol. 42*, 1–29.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Lymn, R. W., & Taylor, E. W. (1971) *Biochemistry 10*, 4617–4324.
Mannherz, H. G., Schenck, H., & Goody, R. S. (1974) *Eur. J. Biochem. 48*, 287–295.
Marston, S., & Weber, A. (1975) *Biochemistry 14*, 3868–3873.
Marston, S. B., Roger, C. D., & Tregear, R. (1976) *J. Mol. Biol. 104*, 263–276.
Maruyama, K., & Weber, A. (1972) *Biochemistry 11*, 2990–2998.
Monod, J., Wyman, J., & Changeux, J. P. (1965) *J. Mol. Biol. 12*, 88–118.
Murray, J. M. (1973) Ph.D. Thesis, University of Pennsylvania.
Murray, J. M., & Weber, A. (1981) in *Regulation of Muscle Contraction: Exitation-Contraction Coupling* (Grinnell, A. D., & Brazier, M. A. B., Eds.) pp 261–275, Academic Press, New York.
Murray, J. M., Weber, A., & Bremel, R. D. (1975) in *Calcium Transport in Contraction and Secretion* (Carafoli et al., Eds.) pp 489–496, Elsevier/North-Holland, Amsterdam and New York.
Murray, J. M., Weber, A., & Wegner, A. (1980a) in *Muscle Contraction; Its Regulatory Mechanisms* (Ebashi, S., Maruyama, K., & Endo, M., Eds.) pp 221–236, Japan Scientific Society Press, Tokyo.
Murray, J. M., Knox, M. K., Trueblood, C. E., & Weber, A. (1980b) *FEBS Lett. 114*, 169–173.
Murray, J. M., Weber, A., & Knox, M. K. (1981) *Biochemistry 20*, 641–649.
Parry, D. A. P., & Squire, J. M. (1973) *J. Mol. Biol. 75*, 33–35.
Pemrick, S., & Weber, A. (1976) *Biochemistry 15*, 5193–5198.
Porter, M., & Weber, A. (1979) *FEBS Lett. 105*, 259–262.
Potter, J. D. (1974) *Arch. Biochem. Biophys. 162*, 436–441.
Sleep, J. A., & Hutton, R. L. (1978) *Biochemistry 17*, 5423–5430.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103–164.
Taylor, R. S., & Weeds, A. G. (1976) *Biochem. J. 159*, 301–315.
Thomas, D. D., Seidel, J. C., & Gergely, J. (1979) *J. Mol. Biol. 132*, 257–273.
Trentham, D. R., Eccleston, J. F., & Bagshaw, C. R. (1976) *Q. Rev. Biophys. 9*, 217–281.
Wagner, P. D., & Giniger, E. (1981) *Biophys. J. 33*, 2329.
Weber, A., & Murray, J. M. (1973) *Physiol. Rev. 53*, 612–673.
Wegner, A. (1979) *J. Mol. Biol. 131*, 839–853.
Wells, J. A., Werber, M. M., & Yount, R. G. (1979) *Biochemistry 18*, 4800–4805.
White, H. D., & Taylor, E. W. (1976) *Biochemistry 15*, 5818–5826.
Wolcott, R. G., & Boyer, P. D. (1974) *Biochem. Biophys. Res. Commun. 57*, 709–716.
Yount, R. G. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 1–56.
Yount, R. G., Ojala, D., & Babcock, D. (1971) *Biochemistry 10*, 2490.

Chemotaxis in *Bacillus subtilis*: Effects of Attractants on the Level of Methylation of Methyl-Accepting Chemotaxis Proteins and the Role of Demethylation in the Adaptation Process†

Daniel J. Goldman, Stephen W. Worobec, Rebecca B. Siegel, Roger V. Hecker, and George W. Ordal*

ABSTRACT: By performing in vivo methylation experiments and using highly resolving $NaDodSO_4$–polyacrylamide gels, we have examined the effects of amino acid attractants on the methylation profile of *Bacillus subtilis* MCPs. Both increases and decreases have been found to occur in the level of methylation of these proteins. By using competition experiments and Conway diffusion cells, we have found that the demethylation event is correlated with the adaptation process. Gas chromatographic analysis indicates that methanol is evolved upon demethylation of these proteins. As more attractant receptors are titrated, corresponding increases in methanol evolution result. During this period of increased rate of methanol production, bacteria swim smoothly.

Bacterial chemotaxis is a primitive sensory system. It is the process by which bacteria sense chemicals in their environment and respond to them by migrating up attractant gradients or down repellent gradients. Bacteria in isotropic medium alternately swim smoothly and tumble. Swimming is correlated with counterclockwise rotation of the flagella (as viewed down the flagellum toward the cell body), and tumbling is correlated with clockwise rotation of the flagella (Macnab, 1978; Silverman & Simon, 1974). When a chemical attractant is added to a suspension of bacteria, one observes an increase in the length of time that the bacteria swim (Berg & Tedesco, 1974). The opposite occurs upon addition of repellents. After this initial response, the bacteria resume their prestimulus behavior even though the chemical stimulus is still present. This phenomenon is referred to as adaptation. In the Gram-negative bacterium *Escherichia coli*, the adaptation process has been correlated with the methylation of certain intrinsic membrane proteins known as methyl-accepting chemotaxis

† From the School of Basic Medical Sciences and Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received July 30, 1981.*

0006-2960/82/0421-0915$01.25/0