Biochemical kinetics of skeletal actoSubfragment-1 at high subfragment-1 concentrations

Leonard A. Stein and Vijay A. Harwalkar
Laboratory of Molecular Cardiology, Departments of Medicine and Physiology, State University of New York at Stony Brook, Stony Brook, New York 11794

ABSTRACT The actomyosin ATPase activity of skeletal myosin subfragment-1 (S-1) is typically studied by keeping the S-1 concentration low and varying the actin concentration. General agreement exists over the kinetic data observed.

Another way of studying the ATPase activity is to keep the actin concentration low and vary the S-1 concentration. The picture that has emerged is that the maximal ATPase rate (per micromolar actin), V^amax, is several fold greater than the V^smax measured at fixed S-1. Likewise, the apparent activation constant K^a_m is several fold weaker than K_{ATPase}. In addition it is found that K^a_m, henceforth $K^a_m(A_t)$, varies with the total actin concentration A_t, but controversy continues over the actin dependence of V^amax. Of particular interest is the fact that the Lymn-Taylor and refractory state models could not account for the data.

Here we have repeated studies on the ATPase activity at fixed actin concentration in an attempt to determine if the current models for the actin activated myosin ATPase activity can account for both the constant actin and constant S-1 data simultaneously, or if these data imply that new kinetic models need be postulated. We conclude that the current kinetic models can account for the data.

INTRODUCTION

Muscle shortens as a result of the sliding of actin filaments relative to myosin filaments, and the process is driven by the interaction of the myosin crossbridges with actin which leads to the hydrolysis of ATP. In order to better understand the actin-myosin interaction from a molecular point of view, biochemists have turned to the kinetics of the actomyosin ATPase activity. The kinetic models resulting from these studies can be expected to be fundamental to the understanding of muscle contraction in vivo.

The usual method used to study the steady state actomyosin ATPase activity is to keep the S-1[1] at low concentrations (i.e., typically <1 μM) and vary the actin over a broad range. The double reciprocal plot of ATPase activity v.s. the actin concentration is linear over a broad range of actin and leads to the extrapolation of a maximum ATPase rate per μM S-1, V^smax, and a Kapparent for the process, usually referred to as K_{ATPase} (Eisenberg and Moos, 1967; Stein, 1988). By definition, K_{ATPase} is the actin concentration where half V^smax is achieved. Numerous studies have been published that estimate V^smax and K_{ATPase} as described above under various conditions of ionic strength, temperature, etc., and these estimates have formed the basis for other biochemical and physiological studies (Lymm and Taylor, 1970, 1971; Eisenberg and Greene, 1980; Brenner and Eisenberg, 1986; Eisenberg and Hill, 1985).

On the other hand there has been a paucity of studies on the kinetics of actoSubfragment-1 where actin is held fixed and S-1 is varied. The published papers that have reported this version of the actomyosin kinetics have been divided on how the Kapparent, $K^a_m(A_t)$, and V^amax determined by extrapolation of the double reciprocal plot of ATPase activity per actin residue v.s. the S-1 concentration, depend on the actin concentration used. It should be noted, that the superscript a in $K^a_m(A_t)$ is meant to imply that this kinetic constant is determined from studies carried out at a fixed total actin concentration, and the A_t in parentheses denotes the actin concentration used. Most of the studies reported appear to agree that the $K^a_m(A_t)$ varies as a function of the actin concentration, but there is division over whether V^amax is constant or also varies with the actin concentration (Eisenberg and Keilley, 1972; Marston, 1978; Wagner et al., 1979). In addition it was found that V^amax was considerably larger than V^smax, a fact that appeared to be inconsistent with the model initially proposed by Lymn and Taylor (1971).

[1]Abbreviations used in this paper: S-1 = myosin subfragment-1; [A] = free actin concentration; V^smax = the maximal ATPase rate per myosin residue; V^amax = the maximal ATPase rate per actin residue; K_{ATPase} = the actin concentration where half the maximal ATPase rate (V^amax) is achieved; K^am(A_t) = the actin concentration where half the maximal rate V^amax is achieved, as a function of the actin concentration; K_b = K(binding) = actin concentration necessary to achieve half S-1 bound, during steady state hydrolysis of ATP. M = myosin subfragment-1; M_t = total S-1 present; A_t = total actin present; D = ADP; T = ATP; Pi = inorganic phosphate; R = ratio of K(binding) to K_{ATPase}, also a dummy variable during curve fitting; (A-1)S-1 and (A-2)S-1, isoforms of skeletal S-1; I.S. = ionic strength.

0006-3495/89/08/263/10 $2.00

In this model, after ATP binding, rapid and complete dissociation of the actomyosin complex occurs. After dissociation, rapid hydrolysis occurs on the free S-1, and this is followed by the rebinding of actin to the myosin-products complex and the rate limiting release of the products of hydrolysis. Because only the product release step contributes to rate limitation in this model, it is predicted that $V^s\text{max} = V^a\text{max}$, and that $K^a_m(A_t) = K_{ATPase}$. Experimentally, however, there was a several fold difference between $V^a\text{max}$ and $V^s\text{max}$ and between $K^a_m(A_t)$ and K_{ATPase} (Eisenberg and Keilley, 1972).

Eisenberg and Keilley (1972) attempted to explain the discrepancy between $V^a\text{max}$ and $V^s\text{max}$ by placing an additional step into the Lymm-Taylor scheme. In the new scheme the rate limiting step occurred in a special transition that followed hydrolysis, and preceded the rebinding of actin, and product release was assumed to be very fast. As a result the new model could qualitatively explain how $V^a\text{max}$ and $V^s\text{max}$ could be different. However, the model also assumed that no actin-S-1 binding occurred at steady state, and as a result the predicted $K^a_m(A_t)$ would be very weak.

In a recent study Rosenfeld and Taylor (1984) attempted to show that the high S-1 data (i.e., constant actin) was consistent with the four state model (see Fig. 1 *a*). In this work data were obtained by varying both the S-1 and actin, but keeping the ratio of actin to S-1 constant. They reported that the apparent activation constant, Km, and the maximal ATPase rate per μM actin, Vmax, measured in these experiments varied as a function of the actin to S-1 ratio.

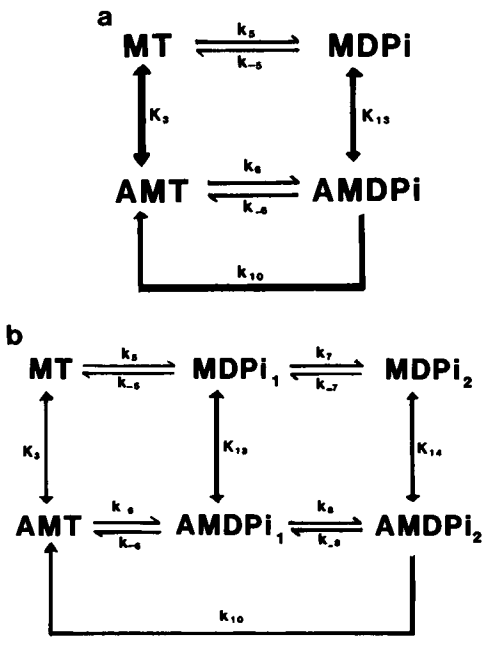

FIGURE 1 The four and six state models. In this figure the dissociation constants $K3$, $K13$, and $K14$ are assumed to represent rapid equilibria. (*a*) Four state model; (*b*) Six state model.

None of these prior studies has adequately accounted for the high S-1 kinetics in a quantitative fashion, and it remains unclear whether the fixed actin and high S-1 kinetic data are consistent with the current models for actin activation shown in Fig. 1. Here we have reinvestigated the kinetics of ATP hydrolysis at fixed actin concentrations in order to determine if the currently accepted models can account for the data. Using a simple kinetic analysis based on these models (see appendix) we have shown that the relationship of the various kinetic constants is given by:

$$V^a\text{max} = (K_b/K_{ATPase})V^s\text{max}$$

and

$$K^a_m(A_t) = K_b + A_t[(K_b/K_{ATPase}) - 1],$$

where K_b is the apparent dissociation constant for the binding of S-1 to actin in the presence of ATP, and A_t is the total actin.

Our experiments show that the maximum hydrolysis rate, $V^a\text{max}$, is within experimental error independent of actin, and that $K^a_m(A_t)$ shows a simple linear dependence on the actin concentration as predicted by the above relations.

METHODS AND MATERIALS

Proteins

Actin, myosin, and subfragment-1 were prepared as described previously (Stein et al., 1979). Standard polyacrylamide gels were performed to monitor the digestion of myosin and the purity of the proteins, as well as to determine the relative ratio of the light chains present. We found that the ratio of (A-1)S-1 to (A-2)S-1 (Weeds and Taylor, 1975) could be enhanced by using only the whitest paraspinal back muscles of the rabbit. In our densitometric assays the ratio of (A-1) to (A-2) has been slightly greater than two after correction for the difference in molecular weight.

Binding assay

The binding assay used is similar to the airfuge technique described previously (Chalovich et al., 1984), except that the pelleting of bound species was carried out in a temperature controlled preparative ultracentrifuge using adapters for 0.3 ml centrifuge tubes. The ionic conditions used for the binding assays can be found in the appropriate figure legends.

ATPase assays

The ATPase assays were carried out as described previously using (gamma-^{32}P) ATP and extracting the hydrolyzed radioactive inorganic phosphate (Stein et al., 1984). In the case of high S-1 kinetics, the rate of hydrolysis was corrected for the rate of hydrolysis in the absence of actin before dividing by the actin concentration. This correction to the

ATPase was often quite large (up to a maximum of 35% of the total measured rate) at the high S-1 concentrations. The ionic conditions of the ATPase assays can be found in the appropriate figure legend.

Data analysis

The binding and ATPase data were analyzed using a hyperbolic fitting procedure, (Brown and Dennis, 1972). The same fitting procedure was also used when analyzing the high S-1 kinetic data in Fig. 4, *a* and *b*. In Fig. 4, *c* and *d*, however, all four sets of data are fit to one V^amax, and one value of R according to the equation: $V = V^a\text{max}[\text{S-1}]/\{K^a_m(A_t) + [S\text{-}1]\}$, where $K^a_m(A_t) = \{K^a_m(0) + (\text{R-1})A_t\}$. The best fit of all the data in Fig. 4, *c* and *d* (as well as each set of data in Table 1) to this equation, leads to the determination of the constants R, V^amax, and $K^a_m(0)$ which characterize the data.

RESULTS

Fig. 2 *a* shows a polyacrylamide gel of the skeletal myosin used. The different columns represent increasing myosin

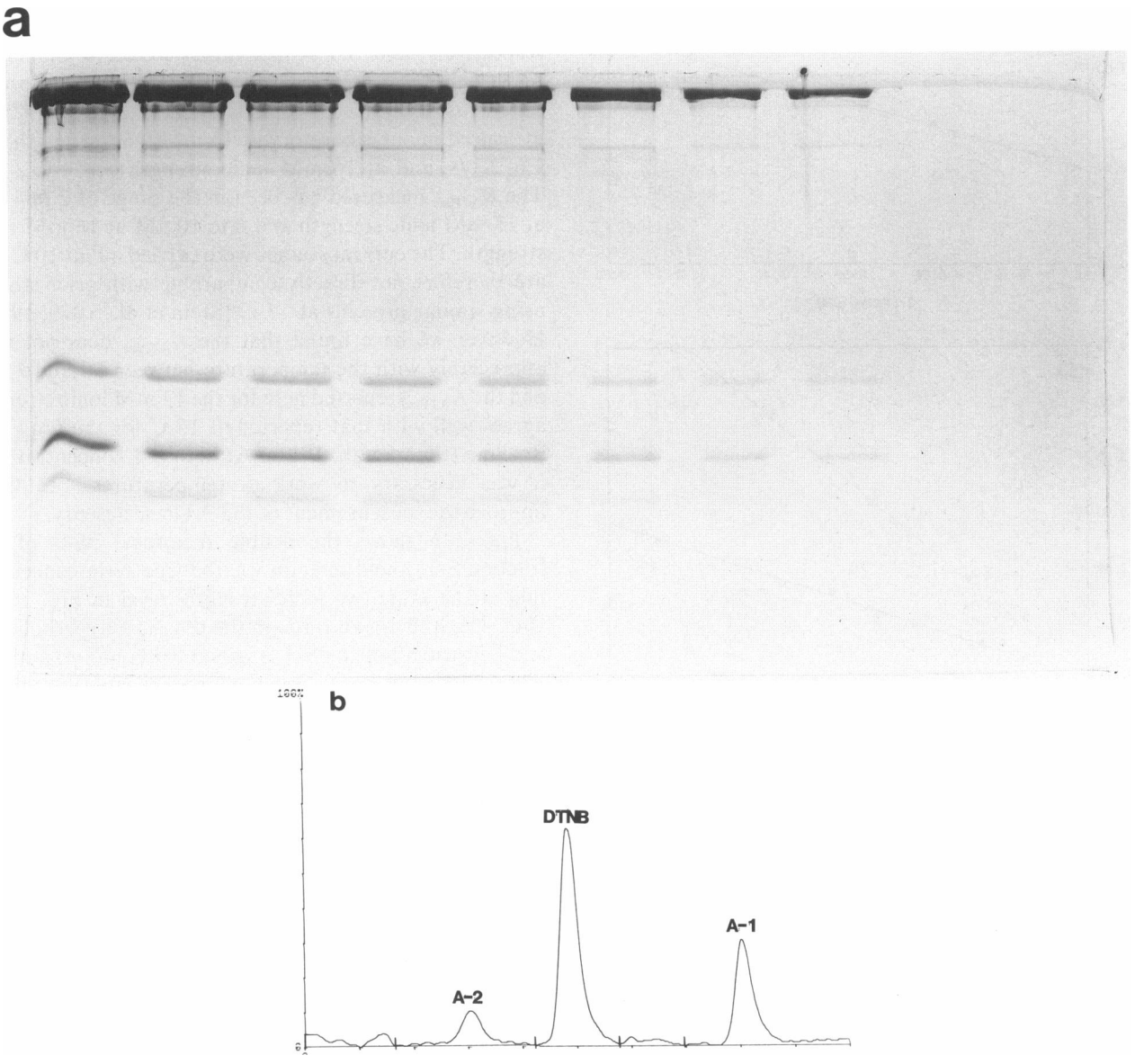

FIGURE 2 (*a*) 14% Polyacrylamide gel of Rabbit skeletal myosin (14% gel is used for the purpose of best separating out the light chains). The rows differ by the concentrations of myosin used, and the relative amounts are given by: 1, 2, 4, 6, 8, 10, 12, 14. The three light chains can be seen to have mass in the order: DTNB > (A-1) > (A-2). (*b*) In this figure a densitometric assay is exhibited of the gel in Fig. 1 *a*. The ratio of the (A-1) band to the (A-2) band is ~2.5. The DTNB band has a density that is approximately equal to the sum of the densities of the other two bands.

concentrations. As can be seen the majority of the skeletal myosin appears to be (A-1)S-1 with a smaller amount of the A-2 light chain present. A densitometric study used to estimate the ratio of A-1 to A-2 is exhibited in Fig. 2 *b*. The ratio of A-1 to A-2 in these preparations is ~2.5/1, and therefore ~70% of the S-1 prepared from this myosin will be (A-1)S-1. It is necessary to use myosin for the determination of the ratio of A-1 to A-2 light chains because when S-1 is purified from myosin after chymotryptic digestion, other digested fragments separate on polyacrylamide gels along with the light chains making any quantitative assessment of the ratio of the light chains less accurate (Wagner et al., 1979).

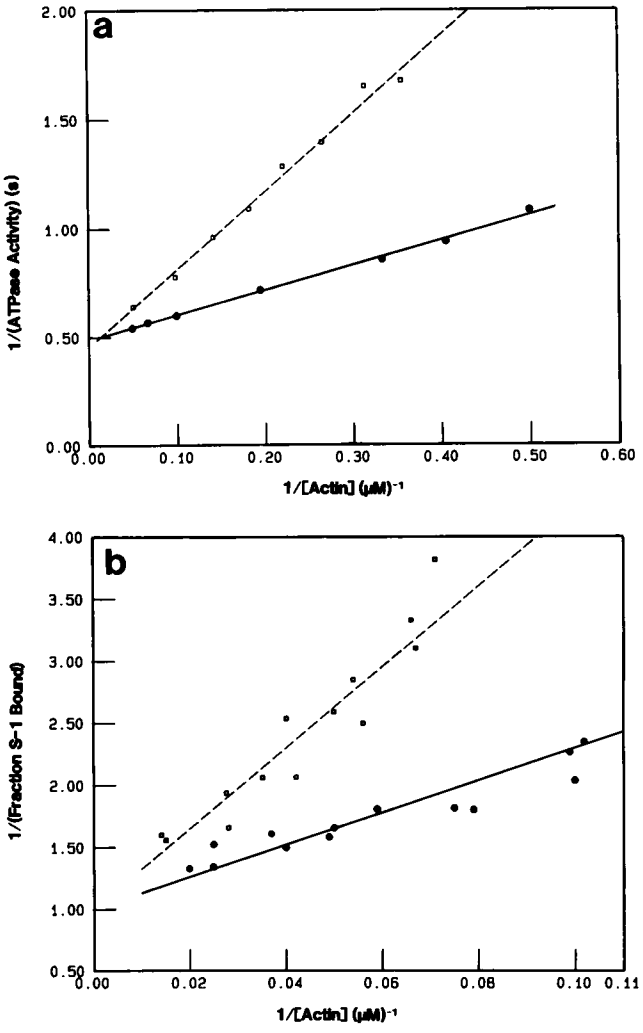

FIGURE 3 Determination of K_{ATPase}, V^smax, and K(binding) (*a*) The ATPase activity per S-1 residue is plotted v.s. the actin concentration at 10 C and two different ionic strengths. Data: ●, 13 mM and □, 16 mM. The kinetic constants are V^smax = 2/s and K_{ATPase} = 2.5 μM at 13 mM ionic strength and V^smax = 2.2/s and K_{ATPase} = 8 μM at 16 mM ionic strength. Conditions: 13 mM ionic strength: 10 mM Imidazole, pH 7.0, 2 mM $MgCl_2$, 1 mM ATP, 1 mM DTT. 16 mM ionic strength: as above except with 3 mM $MgCl_2$. (*b*) The binding of S-1 to actin during steady state hydrolysis of ATP at 10 C, using temperature controlled airfuge technique. ●, 13 mM ionic strength; □, 16 mM ionic strength. The data points come from three different experiments on three distinct protein preparations. The lines drawn through the data represent a hyperbolic fit to the data. The dissociation constants K(binding), are 13 μM at 13 mM ionic strength and 32 μM at 16 mM ionic strength. Conditions as in Fig. 3 *a*.

Fig. 3 *a* shows the double reciprocal plot of the actin activated subfragment-1 ATPase activity per S-1 residue v.s. the actin concentration for a fixed S-1 concentration of 0.5 μM, at 10 C, performed both at 13 mM ionic strength, and at 16 mM ionic strength (see figure captions for conditions). The K_{ATPase} values were 2.5 μM and 8 μM respectively, and the V^smax values were 2.0 and 2.2/s, respectively. To fit the data, a hyperbolic fitting procedure was used (see Methods). The range of V^smax measured in our laboratory at 13 mM ionic strength was 1.6–2.1/s, and at 16 mM ionic strength was 1.9–2.2/s. The K_{ATPase} measured has been in the range of 2 to 4 μM at 13 mM ionic strength and 6 to 10 μM at 16 mM ionic strength. The current studies were carried out at 10 C and are therefore not directly comparable with prior studies using similar proteins at 15 C (Stein et al., 1979, 1981). However we have found that the K_{ATPase} does not vary appreciably with the temperature between 10 and 15 C, and the K_{ATPase} reported here for the 13 mM ionic strength agrees well with that reported at 15 C for the (A-1)S-1 isozyme (Stein et al., 1981). At high S-1 concentrations, it was necessary to work at temperatures <15 C to improve the measurement of the ATPase activity.

Fig. 3 *b* shows the double reciprocal plots of the fraction S-1 bound to actin v.s. the free actin concentration at the same two ionic strengths used in Fig. 3 *a* at 10C. The line drawn through the data is a hyperbolic fit, and because all of the S-1 is presumed bound to actin at infinite actin, we have chosen to force the plot through 1.0 as in our prior studies (Chalovich et al., 1984; Stein and White, 1987; Stein, 1988). The K(binding) is 13 μM at 13 mM ionic strength and 32 μM at 16 mM ionic strength.

The data in Fig. 3, *a* and *b* show that K_{ATPase} and K(binding) differ by approximately a factor of 5 at 13 mM ionic strength and 4 at 16 mM ionic strength. These data agree with previously reported studies at 15 C where the binding and activation constants differed by a factor of 4 to 8 for the subfragment-1 isozyme (A-1)S-1 (Stein et al., 1981). A large difference between K_{ATPase} and K(binding) has also been observed with cardiac proteins (Stein and White, 1987), and is likely to be a general property of the system.

In Fig. 4, *a* and *b* is shown the kinetic data obtained when the actin concentration is fixed and the S-1 concentration is varied, at 13 mM ionic strength and at 10 C. Data are shown for four different actin concentrations:

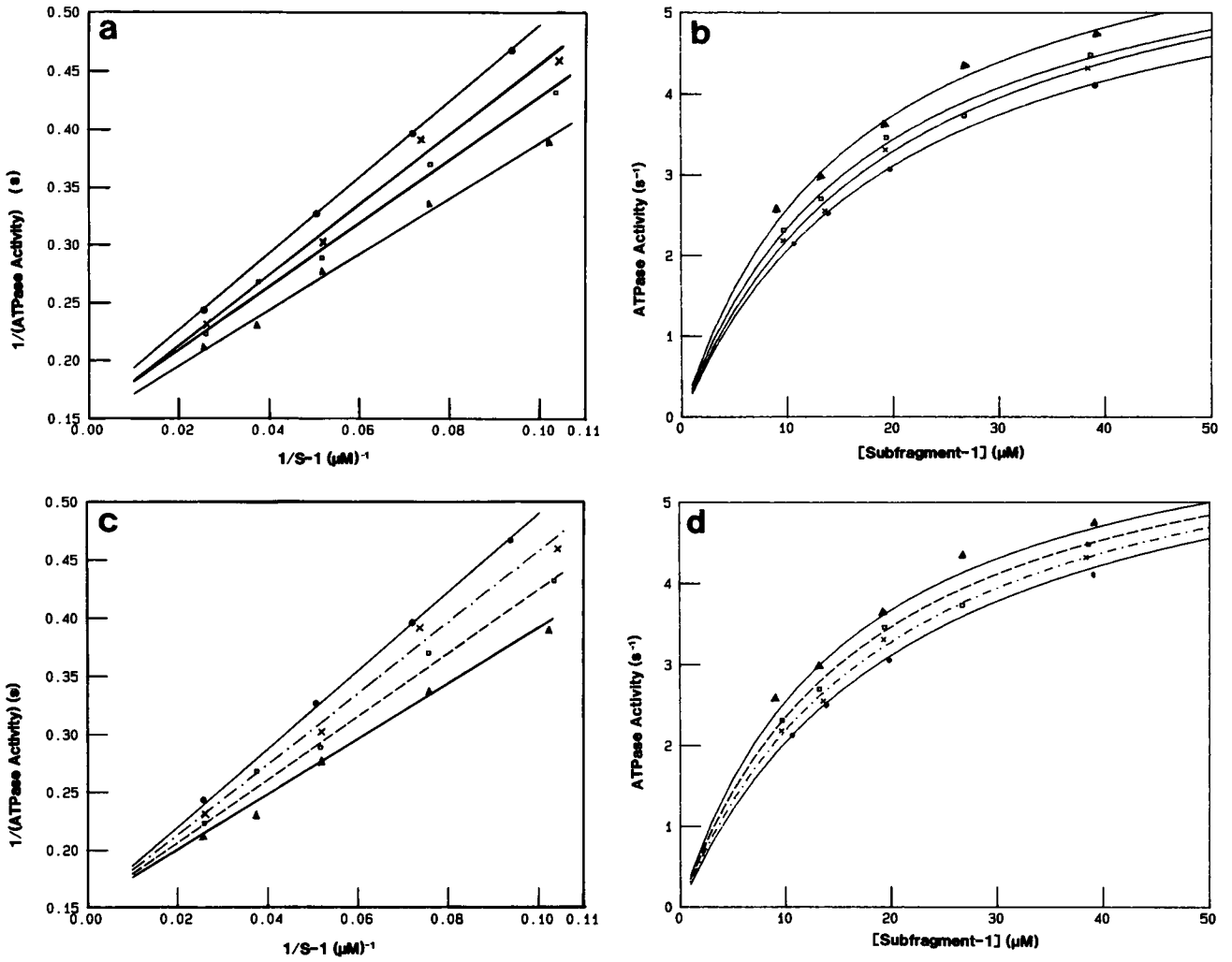

FIGURE 4 High S-1 kinetics at low ionic strength. (*a* and *b*) In this figure the data are plotted, and each set data taken at one particular actin concentration are individually fit to a hyperbola. In *a* the data are plotted in the double reciprocal fashion, and in *b* the data are plotted directly. Data: ▲, 0.5 μM actin; □, 1.0 μM actin; X, 1.5 μM actin; ●, 2 μM actin. The V^amax and $K^a_m(A_t)$ for the actin concentrations of 0.5 μM; 1.0 μM; 1.5 μM; 2.0 μM are given by 6.8/s, 16.4 μM; 6.5/s, 17.8 μM; 6.6/s, 20.1 μM; 6.3/s, 20.5 μM, respectively. Conditions as in Fig. 3. (*c* and *d*) In this figure the same data are plotted, and all the data are fitted to a single V^amax and $K^a_m(A_t)$. In *c* the data are plotted in a double reciprocal fashion, and *d* shows the direct plots. Data: ▲, 0.5 μM actin; □, 1.0 μM actin; X, 1.5 μM actin; ●, 2.0 μM actin. The lines drawn through the data represent a family of hyperbola with V^amax = 6.5/s and $K^a_m(A_t) = (13.7 + 4.2 * A_t)$. The value for $K^a_m(0)$, 13.7, is determined from the fit of the data to the equation $K^a_m(A_t) = K^a_m(0) + (R - 1) * A_t)$, and is not dependent on the value of K(binding) measured in Fig. 2 *b*. Conditions as in Fig. 3.

0.5, 1.0, 1.5, and 2.0 μM actin. In Fig. 4 *a* the data are plotted in a double reciprocal fashion and in Fig. 4 *b* they are plotted directly. The lines drawn through the data were determined using a hyperbolic fitting procedure for each of the sets of data individually (see Methods). As can be seen in Table 1 column B, the extrapolated maximum ATPase rate, V^amax, does appear to be approximately constant and is given by 6.55 ± 0.25(s^{-1}). However, $K^a_m(A_t)$ varies in a monotonically increasing fashion from 16.4 μM to 20.5 uM, with actin ranging from 0.5 to 2.0 μM. Fig. 4, *c* and *d* show the results we obtained when we fit all of the data in Fig. 4 *a* using the hyperbolic equation shown in the Methods which requires that V^amax is constant and $K^a_m(A_t)$ be a linear function of the total actin. The fitted values for the $K^a_m(A_t)$ are shown in Table 1 column C. As can be seen in Table 1, the $K^a_m(A_t)$ values determined from either the individual or the combined fitting procedure are in close agreement. In column C of Table 1, the change in $K^a_m(A_t)$ per unit change in actin is 4.2 μM. Hence, referring to Eq. 8 of the

TABLE 1 Comparison of kinetic constants determined from individual and combined hyperbolic fitting procedure

A Actin concentration	B Kinetic constants obtained in Figs. 4 *A* and *B* (individual fitting)		C Kinetic constants obtained in Figs. 4 *C* and *D* (combined fitting)	
uM	$V^a_{max}(s^{-1})$	$K^a_m(A_t)$ *uM*	$V^a_{max}(s^{-1})$	$K^a_m(A_t)$ *uM*
0.5	6.8	16.4	6.5	15.8
1.0	6.5	17.8	6.5	17.9
1.5	6.6	20.1	6.5	20.0
2.0	6.3	20.5	6.5	22.1

Column B shows the V^a_{max} and $K^a_m(A_t)$ determined using individual hyperbolic fitting procedure described in methods. Column C shows results when all of the data are fit to the model of the appendix, using a least squares fitting procedure for all of the points simultaneously.

Appendix, the ratio of K(binding)$(=K_b)$ to K_{ATPase} is predicted to be 5.2, and K_b is predicted to be 13.7 μM. These predictions for K(binding) and the ratio of K(binding)$/K_{ATPase}$ can be compared with the data in Fig. 3, which shows that K(binding) is 13 μM and K_{ATPase} is 2.5 μM, or a ratio of 5.2, and represents very close agreement.

Because it was not possible to work at higher actin concentrations to show further comparison with the model given in the appendix, we turned to a higher ionic strength to show that approximate agreement was not an accident. In Fig. 5 is shown the high S-1 kinetics at 16 mM ionic strength, and 10 C. The best fit to the combined equation for these data leads to a V^amax of 5.4/s, an R of 3.2, and a K^a_m (0) of 24 μM. These predictions for K(binding) and the ratio K(binding)$/K_{ATPase}$ should be compared with the data in Fig. 3, which reveals a K(binding) of 32 μM and a K_{ATPase} of 8 μM, which also shows good agreement.

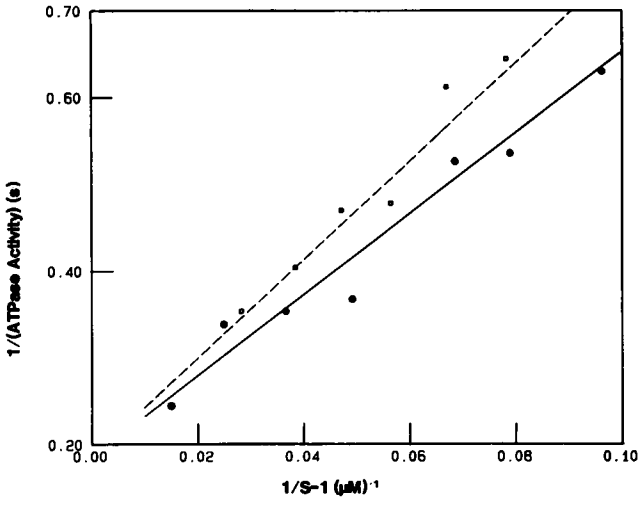

FIGURE 5 High S-1 kinetics at 16 mM ionic strength. In this figure the data are fit to a family of hyperbola with a common V^amax and variable $K^a_m(A_t)$. Data: ●, 0.5 μM actin; □, 3 μM actin. The lines drawn through the data represent a family of curves with a V^amax of 5.4/s and a $K^a_m(A_t) = (24. + 2.2 * A_t)$. Conditions as in Fig. 3.

A summary of the results of four sets of experiments at 13 mM ionic strength is exhibited in Table 2. Note that the $K^a_m(0)$ of the ATPase experiments with actin constant (column B) is in the range of 9 to 14 μM and that this indeed agrees with the K(binding) measured (i.e., 13 μM, Fig. 3). Furthermore, the measured value for R (column B), which can be related to the ratio of K(binding) to K_{ATPase} (see appendix), is in reasonable agreement with the directly measured ratio of K(binding) to K_{ATPase} (see column A of Table 2). Finally, in column C of Table 2 the V^smax and K_{ATPase} are predicted from the data in column B as follows: V^amax $= RV^s$max and $K_{ATPase} = RK^a_m(0)$, and these also show reasonable agreement with the measured values given in column A.

Also in Table 2 is shown three sets of data at 16 mM ionic strength and 10 C. The predicted V^smax and K_{ATPase} (column C) are again in good agreement with the data in column A. However, in this case the $K^a_m(0)$ (column B of Table 2) is somewhat stronger than the measured K(binding) (Fig. 3), being ~22 μM instead of 32 μM. This does not represent a significant problem for the proposed model because the binding predicted is still within the margin of error for the method used.

From the data presented, and the fit of the data to the mathematical scheme proposed, we conclude that the current models for the actin activation of the myosin ATPase activity are consistent with the high S-1 data, and that new models need not be implied.

DISCUSSION

In the studies presented here we have demonstrated that if one works within the proper domains of actin and S-1 concentrations (i.e., domains where the approximations of the appendix are valid), the high S-1 data are consistent with a maximum ATPase activity per actin residue that is independent of the actin concentration, and a variable $K^a_m(A_t)$. Furthermore the ratio of the measured V^amax and V^smax is approximately equal to the ratio of K(binding) to K_{ATPase} as predicted by the theoretical analysis. The data also support a linear relationship between the Kapparent $(K^a_m(A_t))$ for the high S-1 kinetics and the total actin present, another prediction of the model presented in the Appendix.

There are small discrepancies between the observed and predicted kinetic constants as shown in columns A–C of Table 2. One of the major problems is that in order to

TABLE 2 Summary of kinetic data from seven sets of experiments

No. experiment	Ionic strength	A ATPase data (S-1 constant)			B ATPase data (actin constant)			C Predicted kinetic constants	
	mM	V^s_{max}	K_{ATPase}	K_b/K_{ATPase}	V^s_{max}	$K^s_m(0)$	R	V^s_{max}	K_{ATPase}
I	13	2.0	2.5	5.2	6.5	13.6	5.2	1.3	2.6
II	13	2.0	2.4	5.4	8.1	12.7	4.1	2.0	3.1
III	13	2.0	2.0	6.5	6.9	8.5	4.1	1.7	2.1
IV	13	1.9	1.9	6.8	6.7	9.1	3.8	1.8	2.4
V	16	2.0	5.3	6.0	5.9	20.0	3.3	1.7	6.0
VI	16	2.2	8.0	4.0	5.6	25.0*	—	—	—
VII	16	2.2	9.8	3.3	5.4	24.0	3.2	1.7	7.6

Column A: ATPase Data V^s_{max} and K_{ATPase} were determined directly from the double reciprocal plots of the kinetic data. $K_{binding}/K_{ATPase}$ is determined by dividing the K(binding) determined in Fig. 3 *b* by the K_{ATPase} in the table. Column B: ATPase data V^s_{max} and $K^s_m(A_t)$ were determined from the data by fitting the data to a family of hyperbolas with a fixed V^s_{max} and variable $K^s_m(A_t)$, according to $K^s_m(A_t) = [K^s_m(0) + (R - 1)A_t]$.[1] From the data the value of $K^s_m(0)$ and R are determined independently of K(binding) measured in Fig. 3 *b*. According to the model for the high S-1 kinetics presented in the Appendix, $K^s_m(0)$ and K_b are the same. Column C: Predicted rate constants: Using the ATPase data in column B, V^s_{max} and K_{ATPase} can be derived using equations: 1. $K_{ATPase} = K^s_m(0)/R$; 2. $V^s_{max} = V^s_{max}/R$.

[1]In Data set VI, only one set of high S-1 kinetics points were performed at 1uMolar actin. The value given is for K^s_m(Eq. 1).

maintain a low ionic strength, the ATP concentration must be relatively low. The low ATP concentration coupled with the high ATPase activities observed requires that the measurements of the ATPase rate be made at short time intervals, and this increases the error inherent to the experiment especially at the high S-1 concentrations. Furthermore, at the high S-1 concentrations (i.e., 50–100 μM), the correction to the ATPase activity for S-1 alone is quite large (up to 35% of the total rate), and the efficiency of extraction of Pi from the ATPase samples decreases at high protein concentrations, and this too leads to scatter in the data, making the experimental points at the higher S-1 concentration less accurate, and these points strongly influence the extrapolation to a V^smax. These difficulties also limit the range of actin concentrations that can be studied. Within these constraints, however, we feel that the overall agreement between the observed kinetics and the predictions of the model is quite good.

Another possible explanation for the discrepancies seen in Table 2 should also be mentioned. When the kinetics at low and fixed actin concentrations are performed, the actin sites will become saturated at high S-1 concentrations, and this may lead to a perturbation in the expected kinetics due to the available S-1 molecules interfering with each other when they attempt to bind to the actin. This will have the effect that the V^smax measured will be less than that predicted by the model, and may explain why the predicted V^smax in column C is less than that measured directly in column A. However, the discrepancies observed must also be considered to be within the range of experimental error, and without the formalism presented here there would be no way of comparing the data in Columns A and B of Table 2.

As mentioned in the introduction controversy has been present regarding the constancy of V^smax. Eisenberg and Keilley (1972) reported that V^smax was a function of the actin concentration. In our kinetic measurements as shown in Fig. 4 *a*, when the data sets are fit individually to a hyperbolic function, the V^smax is 6.55 ± 0.25 (s^{-1}), in the range of actin from 0.5 to 2.0 μM, and this 3–4% error is not atypical for steady state measurements. We do not know why a varying V^smax was observed by Eisenberg and Keilley (1972), but the data they reported were obtained at only two actin concentrations.

Marston (1978) also studied these high S-1 kinetics at 0 C and 20 C, and also found that V^smax was several times faster than V^smax, and that $K^s_m(A_t)$ varied with the actin concentration. He attempted to explain his data using the Lymm-Taylor model. We find that his data at 0 C and 16 mM ionic strength can be fit to the model presented, with an R (i.e., ratio of K[binding] to K_{ATPase}) of 4–5, and a predicted K[binding] (i.e., K^s_m[0]) of approximately 11 μM. However his 20 C data do not appear to fit well, and this may be a result of the higher temperatures leading to higher rates, greater corrections for S-1 alone, and greater error.

Rosenfeld and Taylor (1984) made the approximation that the ATPase scheme remains essentially in equilibrium at very high S-1/actin ratios, and the model therefore predicts a V^smax and K^s_m that are independent of the actin concentration. These authors performed their experiments by keeping the ratio of S-1 to actin constant, and they found that the double reciprocal plot of the ATPase activity v.s. the S-1 concentration was linear, and that both the apparent activation constant and the maximal ATPase activity varied with the S-1 to actin ratio used until very high ratios, (~50/1), were achieved. The

dependence on the S-1 to actin ratio cannot be accounted for by the equilibrium model proposed, but can be accounted for by the model proposed here (see Appendix).

The model presented here does depend on certain approximations and assumptions. The major assumption used here is that the binding of actin to S-1 during steady state hydrolysis of ATP is described by a single binding isotherm at all actin and S-1 concentrations. In practice when measuring the apparent binding constant, a single isotherm is noted. But in practice when measuring the binding constant, actin is always in excess, whereas in the high S-1 kinetics, it is the S-1 that is in excess. However, the assumption of a single binding isotherm allows us to write an analytical expression for the high S-1 kinetics, which takes into account the subtle changes in the steady state distributions of the kinetic intermediates that occur as a function of the actin concentration (i.e., as the net hydrolysis rate increases). It is these variations of the system from equilibrium that lead to a $K^a_m(A_t)$ that varies as the actin concentration varies.

The formulation of the approximate mathematical scheme in the Appendix is based on only the most fundamental aspects of the four and six state models: the hyperbolic actin dependence of the binding and the ATPase activity. Therefore, it remains interesting to ask whether the high S-1 data is really consistent with both of the current models. Controversy continues to exist over whether the four state model adequately accounts for the existing steady state and presteady state data, and this discrepancy is in part (and largely) due to the differences in K_{ATPase} and K(binding) that are observed experimentally. Rosenfeld and Taylor (1984) find under their conditions (pH 7, 20 C, 15 mM I.S.) that the ratio of K(binding) to K_{ATPase} is in the range of 2 to 3, while Stein and coworkers (Stein, 1988) find a 4–6 fold difference in these kinetic parameters under their usual conditions (pH 7, 10–15 C, 13 mM I.S.). The four state model requires that K(binding) and K_{ATPase} be approximately equal (Stein, 1988), and therefore, it is not surprising that Rosenfeld and Taylor (1974) can obtain a better fit of their data to the four state model than can Stein et al. (1981). However, according to the model presented in the appendix, $K^a_m(A_t)$ changes by $(K_b/K_{ATPase} - 1)$ for each unit change in actin. The data shown in Fig. 4, obtained using the same ionic strength conditions of all prior work, show that $K^a_m(A_t)$ changes by 3–4 μM for each unit change in actin, and in comparison with the model implies that the ratio of K(binding) to K_{ATPase} is in the range of 4–5, which confirms prior estimates of this ratio. This implies, that while the four state model can account for the constant actin data qualitatively, it may have a difficult time accounting for these data quantitatively.

In conclusion the high S-1 kinetics do appear to be consistent with the current models of activation of the myosin ATPase activity. Furthermore the analysis leads to an independent estimate of the apparent binding constant during steady state hydrolysis of ATP.

APPENDIX

In this section we present the theoretical formalism with which to analyze the high S-1 kinetic data. The mathematical expressions that describe the ATPase activity as predicted by the four and six state models have been published previously (Stein, 1988). These mathematical expressions show that the double reciprocal plot of ATPase activity per total S-1 residue v.s. the free actin concentration is linear at low to moderate actin, but then can show a small amount of inhibition at high actin concentrations, and it was the lack of significant inhibition that first led to the suggestion of the four and six state models (Stein et al., 1979). It is not clear, however, if the small amount of inhibition observed is an artifact of the measurement at high actin concentrations, and therefore in the analysis here we shall assume that the ATPase activity can be described by a hyperbola over the range of relevant protein concentrations, and is given by:

$$1/V^s = 1/V^s\text{max} + (K_{ATPase}/V^s\text{max})/([A]), \quad (1)$$

where [A] is the free actin concentration, and K_{ATPase} is the actin concentration required to achieve half V^smax. In this expression, V^s and V^smax are the ATPase activities per μM S-1 present (i.e., the total ATPase activity divided by the total S-1 present). In order to rearrange this expression to the ATPase activity per μM actin, it is necessary to multiply this expression by the total actin present, A_t, and divide by the total myosin subfragment-1 present, M_t. The new steady state rate will be denoted by V^a:

$$1/V^a = (A_t/M_t)/(V^s) = A_t/(M_t V^s\text{max}) + (A_t K_{ATPase}/(M_t V^s\text{max}))/([A]). \quad (2)$$

In order to make further progress a mathematical form for the free actin concentration must be sought. Previous studies have demonstrated that the binding of actin to S-1 during steady state hydrolysis of ATP follows a single binding isotherm (Stein et al., 1979), and it was these studies (in addition to the ATPase studies at high actin concentration) that initially led to the models in Fig. 1. The models themselves, however, have either two (four state model) or three (six state model) actin-S-1 dissociation constants associated with them, and therefore predict a sum of two or three hyperbolae, unless the K's are approximately equal. In fact there is no definitive data which requires that the K's are different, and since the binding data can be fit to a single isotherm (and usually are), and since the analysis using more than one binding isotherm becomes so complex that the results cannot be given analytically (which sacrifices insight) we shall assume that only one binding isotherm is involved, and is given by:

$$(\text{Fraction S-1 bound to actin}) = [A]/([A] + K_b), \quad (3)$$

where K_b (abbreviated from K[binding] for clarity) is the apparent dissociation constant. Assuming a 1 to 1 binding stoichiometry, the actin bound, A_b, equals the S-1 bound that is given by:

$$A_b = (\text{S-1 bound to Actin}) = M_t[A]/([A] + K_b). \quad (4)$$

However, because $A_b = A_t - [A]$, it follows that

$$([A])^2 + ([A])(K_b + M_t - A_t) - A_t K_b = 0. \quad (5)$$

Solving the quadratic and requiring that [A] be positive we find:

$$[A] = [(K_b + M_t - A_t)/2] * (-1 + \{1 + 4A_tK_b/[(K_b + M_t - A_t)^2]\}^{1/2}). \quad (6)$$

The first order approximation is given by (see below):

$$[A] = A_tK_b/(K_b + M_t - A_t). \quad (7)$$

Substituting Eq. 7 into Eq. 2, we find that:

$$1/V^s = 1/V^s\max + (K^s_m(A_t)/V^s\max)/M_t, \text{ where} \quad (8)$$

$$V^s\max = (K_b/K_{ATPase})V^s\max \quad (8a)$$

$$K^s_m(A_t) = K_b + A_t[(K_b/K_{ATPase}) - 1)]. \quad (8b)$$

The analysis therefore suggests that a new $V\max$ ($V^s\max$) will be established and will be equal to the ratio of K_b to K_{ATPase}, multiplied by the $V^s\max$ determined at constant S-1 concentration. Furthermore the new $K^s_m(A_t)$ will be approximately equal to K_b at vanishingly small actin concentrations and increase with increasing actin concentration as long as K_b is weaker than K_{ATPase}.

The approximation made in Eq. 7 should be more carefully examined. The expression we are actually approximating is:

$$-1 + (1 + x)^{1/2} = x/2 - x^2/8 +,$$

where $x = 4A_tK_b/[(K_b + M_t - A_t)^2]$. In order to assure that the first term be a good approximation, we have required that the second term be <3% of the first term. This means that $x/4$ must be <0.03. Under the conditions studied, if A_t is <2.0 μM, K_b is ~13 μM, and M_t >15 μM, then the above expression will be satisfied.

Lymm Taylor and refractory state models

The Lymm-Taylor model, like the four and six state models, predicts that 100% binding will occur at saturating actin with binding constant K_b. In addition it predicts that the K_{ATPase} will equal K_b (Stein 1988). Therefore the same analysis above leads to the equation:

$$1/V^s_{LT} = 1/V^s\max + (K_b/V^s\max)/M_t = 1/V^s_{LT}.$$

Hence the Lymm-Taylor model does not distinguish between the two methods for measuring the ATPase activity because it predicts the same kinetics identically, independent of the protein that is varied. The Refractory state model predicts that no significant binding occurs, and this weak binding predicts a very weak $K^s_m(A_t)$.

High S-1 kinetics at fixed S-1/actin ratio

Rosenfeld and Taylor (1984) performed their experiments at a constant S-1/actin ratio, and it is of interest to determine what the current model predicts under these conditions. Eq. 8 above can be rewritten as follows:

$$1/V^s = (1/V^s\max)[1 + (K_b/K_{ATPase} - 1)(A_t/M_t)] + (K_b/V^s\max)/M_t.$$

The first term is constant at fixed ratios of A_t/M_t, and the plot of $1/V^s$ v.s. $1/M_t$ will be linear. This expression can be placed in the proper hyperbolic form by writing: $1/V = 1/V\max + (Km/V\max)/M_t$ where $V\max = V^s\max/[1 + (K_b/K_{ATPase} - 1)(A_t/M_t)]$ and $Km = K_b/[1 + (K_b/K_{ATPase} - 1)(A_t/M_t)]$.

Both constants will depend on the actin/S-1 ratio used, and will saturate at high S-1/actin ratios.

We wish to thank Ms. Marianne White for her excellent technical assistance, Sankar Narayanan for his assistance in computor-aided data analysis, and Dr. Carl Moos for his critical reading of the manuscript. Furthermore we wish to thank Dr. J. Schmidt of the Biochemistry Department for allowing us to use his densitometer.

The reported research was supported by the National Institutes of Health grant RO1-HL32962, and a Suffolk Heart Association grant both awarded to Leonard A. Stein.

Dr. Stein is the recipient of a Research Career Development Award from the National Institutes of Health.

Received for publication 10 January 1989 and in final form 17 April 1989.

REFERENCES

Brenner, B., and E. Eisenberg. 1986. Rate of force generation in muscle: correlation with actomyosin ATPase activity in solution. *Proc. Natl. Acad. Sci. USA.* 83:3542–3546.

Brown, K. M., and J. E. Dennis. 1972. Derivative free analogues of the Levenberg-Marquardt and Gauss Algorithms for non-linear least squares approximations. *Numer. Math.* 18:289–297.

Chalovich, J. M., L. A. Stein, L. E. Greene, and E. Eisenberg. 1984. Interaction of isozymes of myosin subfragment-1 with actin: effect of ionic strength and nucleotide. *Biochemistry.* 23:4885–4890.

Eisenberg, E., and C. Moos. 1968. The ATPase activity of acto-HMM: kinetic analysis of actin activation. *Biochemistry.* 7:1486–1489.

Eisenberg, E., and W. W. Keilley. 1972. Evidence for a refractory state of HMM and S-1 unable to bind to actin in the presence of ATP. *Cold Spring Harbor Symp. Quant. Biol.* 37:145–152.

Eisenberg, E., and L. E. Greene. 1980. The relation of muscle biochemistry to muscle physiology. *Annu. Rev. Physiol.* 42:921–956.

Eisenberg, E., and T. L. Hill. 1985. Muscle contraction and free energy transduction in biological systems. *Science (Wash. DC).* 227:999–1006.

Lymm, R. W., and E. W. Taylor. 1970. Transient state phosphate production in the hydrolysis of nucleoside triphosphates by myosin. *Biochemistry.* 9:2975–2983.

Lymm, R. W., and E. W. Taylor. 1971. Mechanism of ATP hydrolysis by actomyosin. *Biochemistry.* 10:4617–4624.

Marston, S. B. 1978. Complex kinetics of actin-subfragment-1 ATPase at low temperatures. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 92:147–151.

Rosenfeld, S. S., and E. W. Taylor. 1984. The ATPase mechanism of skeletal and smooth muscle actoS-1. *J. Biol. Chem.* 259:11908–19.

Stein, L. A., R. P. Swartz, P. B. Chock, and E. Eisenberg. 1979. Mechanism of actomyosin ATPase: evidence that adenosine 5′ triphosphate hydrolysis can occur without dissociation of the actomyosin complex. *Biochemistry.* 18:3895–3909.

Stein, L. A., P. B. Chock, and E. Eisenberg. 1981. Mechanism of actomyosin ATPase: effect of actin on the ATP hydrolysis step. *Proc. Natl. Acad. Sci. USA.* 78:1346–1350.

Stein, L. A., P. B. Chock, and E. Eisenberg. 1984. Rate limiting step in the actomyosin ATPase cycle. *Biochemistry.* 23:1555–1563.

Stein, L. A., and M. P. White. 1987. Biochemical kinetics of porcine cardiac subfragment-1. *Circ. Res.* 60:39–49.

Stein, L. A. 1988. The modeling of the actomyosin subfragment-1 ATPase activity. *Cell Biophysics.* 12:29–58.

Taylor, E. W. 1979. Mechanism of actomyosin ATPase and the problem of muscle contraction. *Crit. Rev. Biochem.* 6:103–164.

Wagner, P. D., C. S. Slayter, B. Pope, and A. G. Weeds. 1979. Studies on the actin activation of myosin subfragment-1 isozymes and the role of myosin light chains. *Eur. J. Biochem.* 99:385–394.

Weeds, A. G., and R. S. Taylor. 1975. Separation of subfragment-1 isozymes from rabbit skeletal muscle myosin. *Nature (Lond.).* 251:54–56.