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Rate-Limiting Step in the Actomyosin Adenosinetriphosphatase Cycle: Studies with Myosin Subfragment 1 Cross-Linked to Actin

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ABSTRACT: Although there is agreement that actomyosin can hydrolyze ATP without dissociation of the actin from myosin, there is still controversy about the nature of the rate-limiting step in the ATPase cycle. Two models, which differ in their rate-limiting step, can account for the kinetic data. In the four-state model, which has four states containing bound ATP or ADP·P_i, the rate-limiting step is ATP hydrolysis (A·M·ATP $\rightleftharpoons$ A·M·ADP·P_i). In the six-state model, which we previously proposed, the rate-limiting step is a conformational change which occurs before P_i release but after ATP hydrolysis. A difference between these models is that only the four-state model predicts that almost no acto·subfragment 1 (S-1)·ADP·P_i complex will be formed when ATP is mixed with acto·S-1. In the present study, we determined the amount of acto·S-1·ADP·P_i formed when ATP is mixed with S-1 cross-linked to actin [Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306]. The amount of acto·S-1·ADP·P_i was determined both from intrinsic fluorescence enhancement and from direct measurement of P_i. We found that at μ = 0.013 M, the fluorescence magnitude in the presence of ATP of the cross-linked actin·S-1 preparation was about 50% of the value obtained with S-1, while at μ = 0.053 M the fluorescence magnitude was about 70% of that obtained with S-1. If the ATP hydrolysis step were rate-limiting, the fluorescence magnitude should be less than 10% of that with S-1 alone. Control studies showed that almost none of the fluorescence increase was due to ATP binding. Furthermore, direct measurement of P_i corroborated the fluorescence studies. These results suggest that at both low and high ionic strength, the ATP hydrolysis step is not the rate-limiting step. Instead, the data are consistent with the rate-limiting step occurring before P_i release and after the ATP hydrolysis step, as proposed in the six-state kinetic model.

It is now generally accepted that muscle contraction is driven by the cyclic interaction of the two muscle proteins, actin and myosin, in the presence of ATP. Studies using the soluble proteolytic fragment of myosin, subfragment 1 (S-1),[1] have

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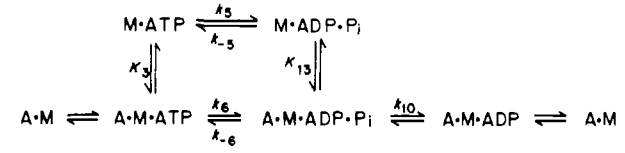
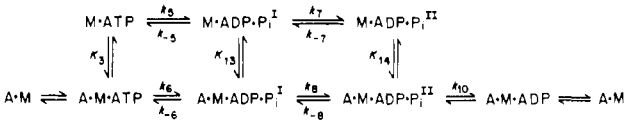
[1] Abbreviations: S-1, subfragment 1 of myosin; acto-S-1, complex of actin with S-1; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MES, 2-(*N*-morpholino)ethanesulfonic acid.

Scheme I: Four-State Model



Scheme II: Six-State Model



clarified the basic elements of the actomyosin ATPase cycle. However, there is still a controversy about the nature of the rate-limiting step in the cycle. This rate-limiting step is important because it may be related to the velocity of muscle contraction (Barany, 1967; Eisenberg et al., 1980).

Two kinetic models which differ only in the nature of their rate-limiting steps have been proposed to account for the properties of the actomyosin ATPase cycle (Stein et al., 1979). The "four-state" kinetic model (Scheme I) has four states containing ATP or ADP·P_i (Stein et al., 1979; Rosenfeld & Taylor, 1984), while the "six-state" model (Scheme II) has six such states (Stein et al., 1979).

We previously showed that in the four-state model, the ATP hydrolysis step (k_6) must be rate limiting, rather than the P_i release step (k_{10}) (Stein et al., 1984). This is because if the P_i release step were rate limiting, K_{ATPase}, the apparent binding constant determined from the double-reciprocal plot of ATPase activity vs. actin concentration, would have to equal $K_{binding}$, the binding constant determined from direct measurement of S-1 binding to actin in the presence of ATP. However, experimental studies (Stein et al., 1979, 1984) clearly show that at 15 °C and very low ionic strength K_{ATPase} is 4–6-fold larger than $K_{binding}$ and at 5 °C this effect becomes even greater (Eisenberg & Kielley, 1972; Wagner & Weeds, 1979). Therefore, under these conditions, the ATP hydrolysis step must be the rate-limiting step. On the other hand, in the six-state kinetic model, both the ATP hydrolysis and P_i release steps are fast, and the rate-limiting step is the transition from M·ADP·P_i^I to M·ADP·P_i^{II} which occurs at about the same rate whether S-1 is bound to (k_8) or dissociated from (k_7) actin.

Since the four- and six-state models differ in their proposed rate-limiting step, they also differ in one major experimental prediction: the magnitude of the P_i burst. In the four-state model, the ATP hydrolysis step (k_6) is rate limiting, and therefore, in this model rapid P_i release will deplete A·M·ADP·P_i much faster than it is formed. This in turn means that the four-state model predicts that there will be almost no initial P_i burst at high actin concentration. On the other hand, in the six-state kinetic model, the rate-limiting step follows the ATP hydrolysis step, and therefore, this model predicts a much larger initial P_i burst at high actin concentration. Our previous experiments (Stein et al., 1981, 1983) using both direct measurements and fluorescence measurements suggested that there is indeed a significant initial P_i burst at high actin concentration. Therefore, these data favored the six-state kinetic model.

In the present study, we reinvestigated the magnitude of the initial P_i burst using S-1 chemically cross-linked to actin by the method of Mornet et al. (1981). The ATPase activity of this cross-linked actin·S-1 is very close to the maximal actin-activated ATPase rate of S-1 (V_{max}). Therefore, S-1 cross-linked to actin appears to act kinetically as if it were in the presence of an infinite concentration of actin. Our results show that when cross-linked actin·S-1 is mixed with ATP, it shows a relatively large initial P_i burst. Assuming that cross-linked S-1 and unmodified S-1 are indeed kinetically identical, these data suggest that the ATP hydrolysis step is not rate limiting at infinite actin concentration and therefore support the six-state model. A preliminary account of this work has been reported (Stein et al., 1983).

Materials and Methods

Rabbit skeletal myosin, S-1, and actin were prepared as described previously (Stein et al., 1979). The molecular weights used for S-1 and actin were 120 000 and 42 000, respectively. Protein concentrations were determined by UV absorption as described previously. To prepare ^{14}C-labeled S-1, the rabbit myosin was modified with iodo[^{14}C]acetamide according to Greene & Eisenberg (1980) and then digested by chymotrypsin to make S-1.

To prepare cross-linked actin·S-1, S-1 was first mixed with iodo[^{14}C]acetamide-modified S-1 (20:1 ratio) to give a total S-1 concentration of ~10 mg/mL and then dialyzed overnight against 10 mM KP_i and 1 mM dithiothreitol, pH 6.8. The specific activity of the S-1 mixture was $\sim 2 \times 10^{12}$ cpm/mol. Before being cross-linked, the F-actin (~4 mg/mL) was dialyzed overnight against 4 mM KP_i and 2 mM $MgCl_2$, pH 6.8. The cross-linking of S-1 to actin was slightly modified from the procedure of Mornet et al. (1981). EDC from a freshly made stock solution of 0.75 M EDC was added to a solution of 66 μM actin in 50 mM MES (pH 6.0) at 20 °C to give a final concentration of 15 mM EDC. After 1 min, 9 volumes of 7.7 μM S-1 in 10 mM MES and 1 mM dithiothreitol was added to the EDC–actin solution. Ten minutes after the start of the reaction, the reaction was quenched by dissociating the S-1 from actin with 4 mM $MgPP_i$ in 0.2 M KCl (pH 7.0). The reaction mixture was centrifuged (2 h, 80000g), and the pellet was washed twice with 10 mM imidazole and 1 mM dithiothreitol (pH 7.0) and then homogenized in 10 mM imidazole and 1 mM dithiothreitol (pH 7.0). To remove any S-1 trapped in the pellet, 4 mM $MgPP_i$ and 0.2 M KCl were again added to the cross-linked preparation, and it was recentrifuged. The resulting pellet was homogenized in 10 mM imidazole and 1 mM dithiothreitol and dialyzed overnight against this same buffer. The cross-linked actin·S-1 preparation was treated 2 times with Dowex (1X-8) to remove residual $MgPP_i$. The amount of free S-1 that remained in the cross-linked preparation after these two dissociation steps was ~5% of the amount of cross-linked S-1, as determined by electrophoresis on 10% sodium dodecyl sulfate–polyacrylamide gels (Laemmli, 1970).

In the actin·S-1 cross-linked preparations, the ratio of cross-linked S-1 to total actin was typically 0.2. Since it is not possible to achieve quantitative recovery of the cross-linked actin·S-1 by resuspending the pellets, this ratio was determined indirectly by subtracting from the total S-1 present the S-1 remaining in the supernatant after sedimentation with $MgPP_i$. The difference gave a measure of the S-1 cross-linked to actin. Both the total S-1 and the S-1 in the supernatant were determined from the specific activity of S-1. It was determined that the S-1 found in the supernatant after sedimention with $MgPP_i$ had the same specific activity as the initial mixture which establishes that the ability of unmodified S-1 and iodo[^{14}C]acetamide-modified S-1 to cross-link to actin is the same. Radioactive samples were counted in a Beckman LS-250 scintillation counter.

Measurements of the rate of fluorescence enhancement and the turbidity of actin·S-1 cross-linked solutions were carried

out in a stopped-flow apparatus as previously described (Stein et al., 1979). For most of the experiments, a specially designed rapid mixing cell was used which had a very low dead time (Rhee & Chock, 1976). This was necessary because of the rapid rate of fluorescence change with cross-linked actin·S-1. The relative magnitude of the fluorescence change which occurred when different samples were mixed with ATP was determined by beginning with the sample with the largest total tryptophan fluorescence, i.e., the cross-linked actin·S-1 sample, and setting the photomultiplier voltage for maximum resolution. This photomultiplier voltage was then held constant when fluorescence measurements were made with samples with less total fluorescence, i.e., S-1 alone. In this way, the magnitude of fluorescence changes with different samples could be compared even though their total fluorescence differed markedly. The validity of this method is confirmed by the experiment shown in Figure 4 where the fluorescence changes which occur with cross-linked S-1 and S-1 alone are shown to be additive.

Quenched flow experiments to determine the P_i burst magnitude were carried out in a Durrum D-132 multimixer as previously described (Chock & Eisenberg, 1979; Stein et al., 1981). Determination of the P_i burst was carried out by extrapolation of the steady-state rate as in our previous publication (Stein et al., 1984) rather than by determination of the early time points as in an earlier publication (Stein et al., 1981). As discussed in detail under Results, the high viscosity of the samples makes mixing slow (Stein et al., 1981), and therefore, measurement of the early time points does not increase the accuracy of the initial P_i burst determination.

In calculating the stoichiometry of the P_i burst, we assumed, as we have in all of our previous papers on this topic (Stein et al., 1981, 1984), that 20% of the added S-1 is incapable of showing an initial P_i burst; i.e., in this respect, it is denatured. This assumption is based on our previous finding that, in the absence of actin, only 80% of the chymotryptic S-1 irreversibly binds ATP (Stein et al., 1981). Therefore, to obtain the equilibrium constant for the ATP hydrolysis step in the absence of actin, we compared the magnitude of the P_i burst with the amount of ATP irreversibly bound to S-1, assuming that only S-1 which irreversibly bound ATP could rapidly hydrolyze ATP in the initial P_i burst. In this way, a value of 0.75 for the initial P_i burst was obtained in the absence of actin. We then applied the same 20% correction in the presence of actin so that the values of the P_i burst in the presence and absence of actin could be directly compared in our theoretical calculations. In the present paper, this same 20% correction was applied, although irreversible binding of ATP could, of course, not be measured with cross-linked actin·S-1.

EDC was from Pierce Chemicals, iodo[^{14}C]acetamide was from Amersham/Searle, and [γ-^{32}P]ATP was from New England Nuclear.

Results

We first used fluorescence measurements to determine whether the cross-linked actin·S-1 preparation shows a significant initial P_i burst. When S-1 is mixed with high concentrations of ATP, almost all of the fluorescence increase which is observed is due to the transition from M·ATP to M·ADP·P_i, that is, to the initial P_i burst (Johnson & Taylor, 1978; Chock et al., 1979). The fluorescence change which is due to ATP binding occurs too rapidly to be observed. Assuming that the transition from A·M·ATP to A·M·ADP·P_i causes the same increase in fluorescence as the transition from M·ATP to M·ADP·P_i, then comparison of the magnitude of the fluorescence increase which occurs with cross-linked actin·S-1 to that with S-1 should reveal whether cross-linked actin·S-1 shows an initial P_i burst.

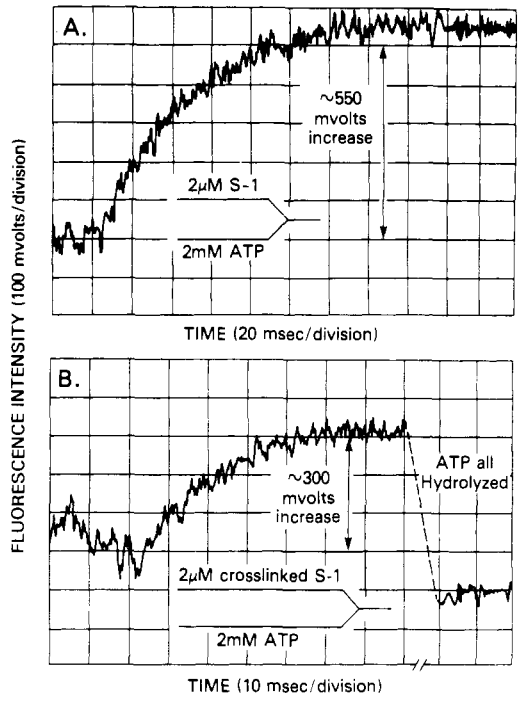


FIGURE 1: Magnitude of the fluorescence change upon mixing ATP with either S-1 or cross-linked actin·S-1 at low ionic strength. Conditions were 1.8 mM $MgCl_2$, 1.0 mM ATP, 1 mM dithiothreitol, and 10 mM imidazole (pH 7.0) at 15 °C. In panel A, 2 μM S-1 was mixed with 2 mM ATP, while in panel B, 2 μM cross-linked S-1 was mixed with 2 mM ATP. Note that the time scales are different.

Previous modeling experiments (Stein et al., 1984) have shown that, for the four-state kinetic model to explain the difference between K_{ATPase} and $K_{binding}$ observed with (A-1)S-1 at very low ionic strength at 15 °C, k_{10}, the P_i release step, has to be more than 20 times larger than k_6, the ATP hydrolysis step (80 s^{-1} compared to 3 s^{-1}). This leads to the prediction that, under these conditions, the fluorescence increase for (A-1)S-1 at inifinite actin concentration, i.e., for cross-linked actin·(A-1)S-1, should only be about 4% of that for (A-1)S-1 alone. A similar effect will occur with unfractionated S-1 since both (A-1)S-1 and (A-2)S-1 show a similar difference between K_{ATPase} and $K_{binding}$ (Chalovich et al., 1984).

Figure 1 shows a comparison of the fluorescence increase of S-1 and cross-linked actin·S-1 at μ = 0.013 M, 15 °C. As can be seen, the fluorescence increase obtained with cross-linked actin·S-1 is more than 50% of the fluorescence increase obtained with S-1, i.e., about 10 times larger than predicted by the four-state kinetic model. Since with S-1 the P_i burst (i.e., the fraction of S-1 which occurs as M·ADP·P_i) is about 0.70, these fluorescence data suggest that about 38% of the cross-linked actin·S-1 occurs as A·M·ADP·P_i. On the basis of our previous direct and fluorescence measurements of the initial P_i burst at high actin concentrations, we would have expected about 45% of the cross-linked actin·S-1 to occur as A·M·ADP·P_i. Our measured value may be slightly low because part of the reaction is lost in the dead time of the stopped-flow apparatus. Evidence that this may be the case is provided by the observation that the decrease in fluorescence which occurs after all of the ATP is hydrolyzed is larger than the increase in fluorescence which occurs at the beginning of the reaction (Figure 1). This effect was observed in many of our samples, perhaps because, as observed previously (Stein et al., 1981, 1984; Marsh et al., 1982), the rate of the fluorescence increase in the presence of actin is more than twice

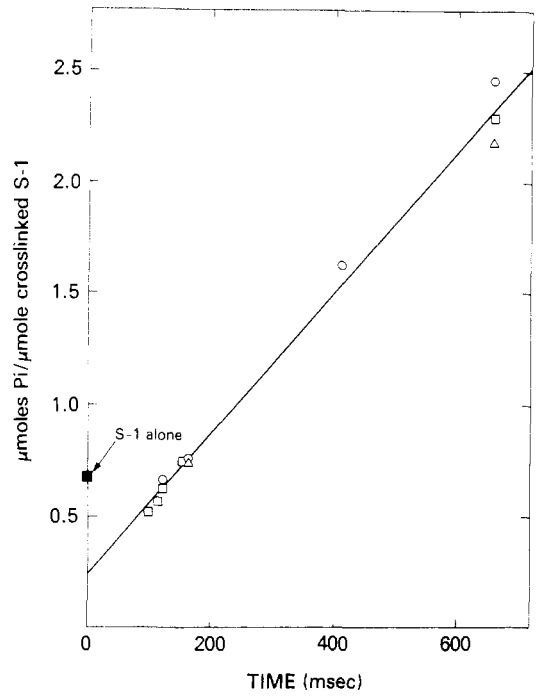


FIGURE 2: Phosphate production obtained with either S-1 or cross-linked actin·S-1 at low ionic strength. Conditions were the same as given in Figure 1, except the ATP concentration was 100 μM. With cross-linked actin·S-1 (open symbols), 20 μM cross-linked S-1 was mixed with 200 μM ATP, and then at the indicated times, 2 N HCl was added to the mixture to quench the reaction. The different open symbols represent different cross-linked actin·S-1 preparations. With S-1 alone (closed square), 20 μM S-1 was mixed with 200 μM ATP. The magnitude of the initial P_i burst obtained from the ordinate intercept is 0.70 for S-1 alone and 0.24 for cross-linked actin·S-1. The steady-state rate of ATP hydrolysis of the cross-linked S-1 is 3.2 s^{-1}. Linear regression analysis was used to obtain the line drawn through the cross-linked actin·S-1 data (correlation coefficient = 0.99).

as fast as the rate in the absence of actin, resulting in a loss of signal when the viscous cross-linked actin·S-1 sample is used. Therefore, the fluorescence experiments on cross-linked actin·S-1 indicate that S-1 shows a relatively large initial P_i burst at high actin concentration, as predicted by the six-state kinetic model.

Although these fluorescence experiments are very suggestive, they do depend on the assumption that the transition from AM·ATP to AM·ADP·P_i shows the same fluorescence increase as the transition from M·ATP to M·ADP·P_i; if the magnitude of the fluorescence increase for the former transition were 10 times greater than for the latter transition, our data would be consistent with the four-state kinetic model rather than the six-state kinetic model. Therefore, we next directly measured the initial P_i burst of cross-linked actin·S-1 using quench-flow, as in our previous study of the initial P_i burst of acto·S-1 (Stein et al., 1979, 1981, 1984). As can be seen in Figure 2, the results of a number of different experiments yielded a value of 0.24 for the initial P_i burst of cross-linked actin·S-1 compared to a value of 0.70 for S-1. The four-state kinetic model predicts that the direct measurement of the initial P_i burst should be less than 0.02, while our previous direct measurements at high actin concentration yielded a value for the initial P_i burst of about 0.35 (Stein et al., 1981, 1984). Note that the measured initial P_i burst is about 5–10% less than the amount of M·ADP·P_i and A·M·ADP·P_i present during steady-state ATP hydrolysis because determination of the initial P_i burst by extrapolation of the steady-state ATPase rate involves subtraction of the steady-state P_i release from the time the proteins are initially mixed (time zero) rather than from the time the steady-state rate actually begins (Stein et al., 1979, 1981, 1984).

This subtraction from time zero causes mixing delays to have an unusually large effect on our measurement of the initial P_i burst. The longer the mixing delay, the longer the delay before the ATPase rate reaches its steady-state value. Since the steady-state rate is subtracted from time zero, this can lead to large errors in the determination of the initial P_i burst. The errors will be particularly large when the magnitude of the initial P_i burst is relatively small because the absolute magnitude of the error depends only on the length of the mixing delay and the steady-state ATPase rate. Therefore, our measurement probably underestimates the value of the initial P_i burst of cross-linked S-1.

The experiments we have described thus far were carried out at very low ionic strength, conditions previously used in our kinetic studies on acto·S-1 to maximize the strength of binding of S-1 to actin. As the ionic strength is increased, this binding becomes considerably weaker (Greene et al., 1983) which, of course, makes it impossible to determine the effect of actin on the S-1 ATPase activity. However, with cross-linked actin·S-1, this problem does not occur; since the S-1 is chemically cross-linked to actin, increasing ionic strength does not dissociate the S-1 from the actin. Therefore, with cross-linked actin·S-1, it is possible to determine whether acto·S-1 has an initial P_i burst at relatively high ionic strength. This has two advantages over the experiments at low ionic strength. First, at high ionic strength, the magnitude of the initial P_i burst of S-1 alone increases as the equilibrium shifts from M·ATP toward M·ADP·P_i (Johnson & Taylor, 1978; Chock et al., 1979). Second, working at high ionic strength makes it possible to perform several important control experiments which tend to rule out the possibility that our measurements of the initial P_i burst are artifactually high. When free S-1 is added to the cross-linked actin·S-1 at high ionic strength in the presence of ATP, it does not interact with the free actin which is present in the cross-linked actin·S-1 preparation and therefore shows a normal initial P_i burst. Hence, it can be determined whether the initial P_i burst of the added S-1 is additive with the initial P_i burst of the cross-linked actin·S-1, as would be expected if our measurement of the initial P_i burst is valid.

Figure 3A,B shows a comparison of the fluorescence increase which occurs when ATP is mixed with cross-linked actin·S-1 and S-1 alone at μ = 0.053 M. As can be seen, at this ionic strength, the fluorescence increase obtained with cross-linked actin·S-1 is about 80% of that obtained with S-1. This is a larger percentage than we observed at very low ionic strength, which suggests that the initial P_i burst increases even more with cross-linked actin·S-1 than with S-1 as the ionic strength is increased.

As we pointed out above, the increase which occurs with S-1 should be directly additive to the increase which occurs with cross-linked actin·S-1. Figure 3 shows that this is indeed the case. When ATP is added to cross-linked actin·S-1 alone, there is about a 400-mV increase (Figure 3A); with S-1 alone, there is about a 500-mV increase (Figure 3B); and when the two are mixed together, there is about a 800-mV increase (Figure 3C). This suggests that there is no mixing artifact occurring which makes the fluorescence increase of the cross-linked actin·S-1 appear too large.

Adding ATP to the mixture of S-1 and cross-linked actin·S-1 also allows us to perform another control. We can make certain that almost all of the fluorescence increase is associated with the ATP hydrolysis step rather than with the actual ATP

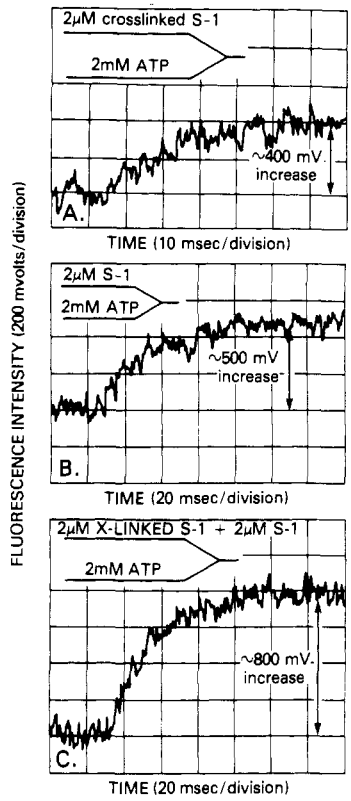


FIGURE 3: Magnitude of the fluorescence change of cross-linked actin·S-1, S-1 alone, or a mixture of cross-linked actin·S-1 and S-1 upon addition of ATP at μ = 0.053 M. The conditions are the same as in Figure 1, except 40 mM KCl was added. In panel A, 2 μM cross-linked S-1 was mixed with 2 mM ATP; in panel B, 2 μM S-1 was mixed with 2 mM ATP; in panel C, 2 μM cross-linked S-1 and 2 μM S-1 were mixed with 2 mM ATP. Note that the time scales are different.

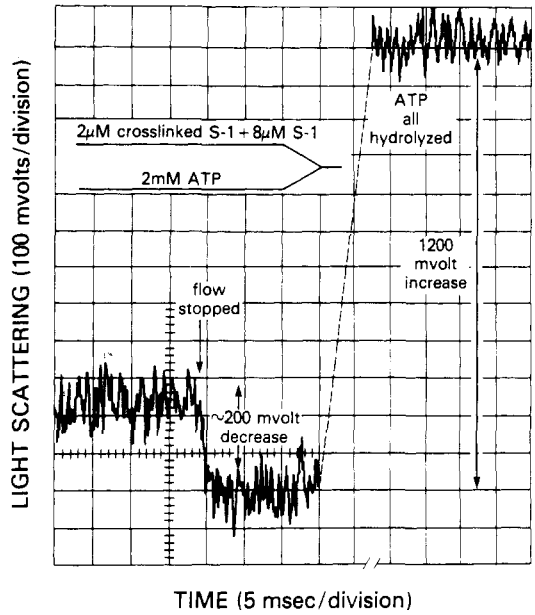


FIGURE 4: Time course of change in light scattering upon mixing 2 mM ATP with a mixture of 2 μM cross-linked S-1 and 8 μM S-1 at μ = 0.053 M. Conditions are the same as those given in Figure 3.

binding step. Figure 4 shows the time course of the light-scattering change which occurs when ATP is added to a mixture of S-1 and cross-linked actin·S-1. This light-scattering change is associated with the dissociation of S-1 from actin and therefore has the same time course as ATP binding (Chock et al., 1979). As can be seen, it occurs much more

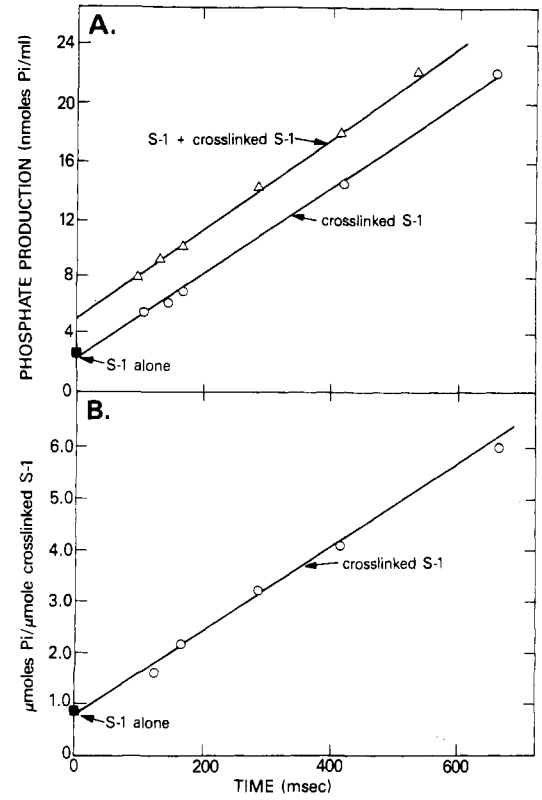


FIGURE 5: Phosphate production obtained with cross-linked actin·S-1 and S-1. In panel A, the phosphate production with S-1, cross-linked actin·S-1, and a mixture of S-1 and cross-linked actin·S-1 was measured at μ = 0.053 M. Conditions are the same as in Figure 2 except 40 mM KCl was added. With S-1 (closed square), 13 μM S-1 was mixed with 200 μM ATP. With cross-linked actin·S-1 (open circles), 20 μM cross-linked S-1 was mixed with 200 mM ATP. With the mixture (open triangles), 20 μM cross-linked S-1 and 13 μM S-1 were mixed with 200 μM ATP. After correction for the 4-fold dilution of the proteins due to mixing, the magnitude of the P_i burst was determined from the ordinate intercepts to be 0.75 and 0.55 for S-1 and cross-linked actin·S-1, respectively. The steady-state rate of ATP hydrolysis of the cross-linked S-1 is 7.5 s^{-1}. In panel B, phosphate production obtained with either S-1 alone or cross-linked actin·S-1 was measured at μ = 0.17 M. Conditions are 153 mM KCl, 100 μM ATP, 1 mM dithiothreitol, and 10 mM imidazole (pH 7.0) at 15 °C. With cross-linked actin·S-1 (open circles), 20 μM cross-linked S-1 was mixed with 200 μM ATP, while with S-1 (closed square), 20 μM S-1 was mixed with 200 μM ATP. The initial P_i burst is 0.87 for S-1 and 0.75 for cross-linked actin·S-1. The steady-state rate of ATP hydrolysis of the cross-linked S-1 is 8 s^{-1}.

rapidly than the fluorescence increase. Assuming that, at high ATP concentration, the rates of ATP binding to acto·S-1 and cross-linked actin·S-1 are identical, these data show that the observed fluorescence increase is indeed almost entirely due to the ATP hydrolysis step.

We next directly measured the phosphate production at μ = 0.053 M using the quench flow technique (Figure 5A). From the ordinate intercepts, the initial P_i burst is calculated to be 0.75 and 0.55 for S-1 and cross-linked actin·S-1, respectively. Therefore, the initial P_i burst of S-1 has increased from 0.70 to 0.75 as the ionic strength changed from 0.013 to 0.053 M. At the same time, the initial P_i burst of the cross-linked actin·S-1 has increased even more, from a value of 0.24 per mol of S-1 at μ = 0.013 M to a value of 0.55 per mol of S-1 at the higher ionic strength. On the basis of our fluorescence measurements, we would have expected a value of about 0.5 per mol of S-1. Therefore, these data are in reasonable agreement with our fluorescence measurements and suggest that cross-linked actin·S-1 shows a relatively large initial P_i burst at μ = 0.053 M.

As we discussed above, the initial P_i burst is difficult to measure directly because of the high viscosity of the sample solution. Therefore, to make certain that our direct measurement of the initial P_i burst is not artifactually high, we were particularly interested in determining whether, in a quench flow experiment at $\mu = 0.053$ M, the initial P_i burst of the S-1 is additive with the initial P_i burst of the cross-linked actin·S-1. Figure 5A shows the phosphate production of a mixture of S-1 and cross-linked actin·S-1 in comparison with that obtained with S-1 and cross-linked actin·S-1 measured individually. As can be seen, the initial P_i burst of the mixture (obtained from the ordinate intercept) is quite close to the theoretical value obtained by adding the initial P_i burst obtained with S-1 and cross-linked actin·S-1. This suggests that we are not overestimating the value of the initial P_i burst. Note, however, that this is not inconsistent with our earlier suggestion that, at very low ionic strength, we are somewhat underestimating the value of the initial P_i burst. As pointed out above, when the initial P_i burst is large, mixing delays have much less effect than when the initial P_i burst is small. Therefore, in the experiment with S-1 and cross-linked actin·S-1 mixed together, mixing delays would be expected to have a relatively small effect.

The ability to measure the initial P_i burst of cross-linked S-1 at high ionic strength allows us to determine its value at physiological ionic strength ($\mu = 0.17$ M). The direct measurement of the initial P_i burst at physiological ionic strength is shown in Figure 5B. The initial P_i burst of the S-1 alone is about 0.85, while the initial P_i burst of the cross-linked S-1 is about 0.75. These data indicate that, under physiological conditions, S-1 bound to actin shows a significant initial P_i burst. In addition, this continues the trend observed at $\mu = 0.053$ M of the cross-linked actin·S-1 showing a larger initial P_i burst as the ionic strength is increased.

Discussion

The evidence presented in this paper in support of the six-state kinetic model was obtained by using S-1 cross-linked to actin. Therefore, we are assuming that cross-linked actin·S-1 has the same kinetic properties as S-1 at infinite actin concentration. Several aspects of our data support this assumption. First, the ATPase activity of the cross-linked actin·S-1 is about 80% of the maximum actin-activated ATPase rate of S-1 (V_{max}) (Mornet et al., 1981; B. Brenner and E. Eisenberg, unpublished results). Second, similar to the V_{max} determined with acto-S-1, the ATPase activity of the cross-linked actin·S-1 shows relatively little change with increasing ionic strength, only about a 2-fold increase over a range of ionic strengths from 0.012 to 0.17 M (B. Brenner and E. Eisenberg, unpublished results). Finally, both by fluorescence measurement and by direct measurement, the cross-linked actin·S-1 appears to show an initial P_i burst, especially at high ionic strength. Therefore, these data support the view that the kinetic behavior of cross-linked actin·S-1 is an accurate reflection of the behavior of S-1 at infinite actin concentration.

In using the cross-linked actin·S-1 to distinguish between the four-state and six-state kinetic models, we made use of the fact that these models differ in one major experimental prediction. The four-state kinetic model predicts that there will be almost no initial P_i burst when S-1 is bound to actin at very low ionic strength (Stein et al., 1984). This is because the ATP hydrolysis step is the rate-limiting step in the ATPase cycle; P_i release cannot be rate limiting because K_{ATPase} is more than 4-fold greater than $K_{binding}$. On the other hand, the six-state kinetic model predicts that there will be a significant initial P_i burst when S-1 is bound to actin (Stein et al., 1981, 1984) because the rate-limiting step follows the ATP hydrolysis step but precedes P_i release.

Our previous quench flow experiments (Stein et al., 1981, 1984) suggested that, even at high actin concentration, S-1 shows a significant initial P_i burst at very low ionic strength. However, we did not consider these experiments conclusive because it is difficult to directly measure the initial P_i burst at high actin concentration. Therefore, in the present study we measured the initial P_i burst of S-1 cross-linked to actin which, in effect, puts S-1 in the presence of an infinite actin concentration. Unfortunately, here too, we cannot consider the results of the direct measurement of the initial P_i burst at very low ionic strength totally unambiguous. We obtained an experimental value of 0.24 mol of P_i per mol of S-1 compared to the theoretical value of less than 0.02 predicted by the four-state model and our previously measured value of about 0.35 with S-1 at high actin concentrations. Note that even the value of 0.24 for the magnitude of the P_i burst is inconsistent with the four-state kinetic model. Such a magnitude requires that $k_6 \simeq k_{-6} \simeq k_{10} \simeq 20\ s^{-1}$ in the four-state model (Scheme I). Figure 4 of our previous publication (Stein et al., 1984) showed that with these values the four-state model cannot account for the observed difference between K_{ATPase} and $K_{binding}$.

Generally, in situations where the kinetic data are ambiguous, it is best to retain the simplest kinetic model, that is, the model with the fewest kinetic states. However, in the present choice between the four-state and six-state kinetic models, several other aspects of the data favor the six-state model. First, there are the fluorescence data obtained at very low ionic strength. Our previous experiments (Stein et al., 1984) showed that the rise in fluorescence which occurs when M·ATP is mixed with actin is about twice the fall in fluorescence which occurs when M·ADP·P_i is mixed with actin, the opposite of the result predicted by the four-state kinetic model. Our current results show that the fluorescence increase which occurs when ATP is mixed with cross-linked actin·S-1 is 10-fold greater than that predicted by the four-state model. They also show that this fluorescence increase occurs after the ATP binding step and follows the time course expected for the ATP hydrolysis step. Therefore, assuming that, at high ATP concentration, all of the observed fluorescence change is indeed due to the ATP hydrolysis step as suggested by both Johnson & Taylor (1978) and Chock et al. (1979), then all of our fluorescence experiments strongly suggest that a significant initial P_i burst occurs when S-1 is bound to actin, which in turn implies that the six-state kinetic model is valid.

The second aspect of our data which favors the six-state model is the measurement of the initial P_i burst at high ionic strength. Both the fluorescence measurement and the direct measurement show that, as the ionic strength is increased, the magnitude of the initial P_i burst of cross-linked actin·S-1 also increases, with the directly measured initial P_i burst reaching a value of 0.75 per mol of S-1 at $\mu = 0.17$ M. This increase in the initial P_i burst indicates a shift in the equilibrium constant between A·M·ATP and A·M·ADP·P_i toward A·M·ADP·P_i; there is a similar shift in the equilibrium constant between M·ATP and M·ADP·P_i as the ionic strength is increased (Johnson & Taylor, 1978; Chock et al., 1978).

The occurrence of a large initial P_i burst unambiguously shows that the ATP hydrolysis step is not rate limiting at high ionic strength. Therefore, even if the ATP hydrolysis step were rate limiting at very low ionic strength, our data suggest that P_i release must become rate limiting as the ionic strength is increased. Furthermore, this change in the rate-limiting step

must occur without causing a large change in the overall ATPase rate of the cross-linked S-1, since this rate only increases about 2-fold as the ionic strength is increased from $\mu = 0.012$ M to $\mu = 0.17$ M. Such a shift in the rate-limiting step is not impossible, but it does require a rather coincidental change in a number of rate constants in the kinetic model. As the ionic strength is increased, the rate of the P_i release step must decrease to the rate of the ATP hydrolysis step at very low strength, while the rate of the ATP hydrolysis step must correspondingly increase. Since such a coincidental shift in the rate-limiting step is not required by the six-state model, in this one respect it is actually simpler than the four-state model.

In this regard, it should be noted that the value of the rate-limiting step apparently plays an important physiological role in the contractile cycle. There is evidence that this step controls the rate at which the cross-bridge develops force during each cycle of ATP hydrolysis (Brenner et al., 1984). Therefore, it may be an important determinant of the velocity of muscle contraction and would not be expected to be determined by coincidental properties of the kinetic model. In summary then, while the direct measurement of the initial P_i burst at low ionic strength yields a somewhat low value (possibly because of poor mixing in the quench flow apparatus), the fluorescence data and the data obtained at high ionic strength support the validity of the six-state model.

As we pointed out in our previous publication, if the six-state kinetic model is valid, it raises a question as to why the ^{18}O exchange, which occurs during ATP hydrolysis by S-1 alone, decreases to zero when S-1 hydrolyzes ATP at very high actin concentration (Sleep & Boyer, 1978). ^{18}O exchange appears to be due to a combination of rotation of the phosphate at the active site and reversal of the ATP hydrolysis step, i.e., the occurrence of the reverse transition from M·ADP·P_i to M·ATP. Since our data show that a significant amount of A·M·ADP·P_i occurs with cross-linked actin·S-1, it might be expected that cross-linked actin·S-1 would show a significant amount of ^{18}O exchange. However, it has been reported that, as with S-1 at high actin concentration, cross-linked actin·S-1 also shows essentially no ^{18}O exchange (Webb & Trentham, 1982). One possible explanation of these data is that although the reverse transition from A·M·ADP·P_i to A·M·ATP occurs with cross-linked actin·S-1, rotation of the phosphate at the active site is inhibited when S-1 is bound to actin. Further work will be necessary to determine if this explanation is valid.

Registry No. ATPase, 9000-83-3; phosphate, 14265-44-2.

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