Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 5345–5349, September 1984
Biochemistry

Catalytic consequences of oligomeric organization: Kinetic evidence for "tethered" acto-heavy meromyosin at low ATP concentrations

(**^{18}O exchange/subunit interactions/myosin ATPase**)

DAVID D. HACKNEY AND PATRICK K. CLARK

Department of Biological Sciences, Carnegie-Mellon University, Pittsburgh, PA 15213

Communicated by Paul D. Boyer, May 14, 1984

ABSTRACT **The influence of the supramolecular organization of myosin on its ATPase activity was investigated at a range of ATP concentrations, using as a model system subfragment 1 (S1) and heavy meromyosin (HMM), which are respectively monomeric and dimeric proteolytic fragments of myosin. At low ATP levels in the presence of a molar excess of actin, dimeric HMM showed an increased rate of ATP hydrolysis relative to that for monomeric S1. This increased ATPase for HMM was inhibited by high concentrations of ATP, which reduced the acto-HMM ATPase rate to the lower level of acto-S1. This observation is consistent with the rapid ATP hydrolysis of acto-HMM at low ATP being due to rapid product release from a "tethered" acto-HMM species, which has product bound to one head group while the other head group remains bound to actin. At high concentrations of ATP, ATP binds to both head groups, resulting in net dissociation of HMM from actin. This model is supported by ^{18}O exchange data. Acto-HMM hydrolyzed ATP with extensive exchange of water oxygens into P_i at high ATP levels, but not at low ATP levels. Acto-S1 exhibited extensive exchange at both high and low ATP levels. This result is consistent with rapid product release from a tethered acto-HMM intermediate at low ATP.**

The possible catalytic significance of the organization of enzymes into supramolecular structures is a problem that is central to the extension of enzymology from the study of reactions in dilute solution *in vitro* into the complexity of reactions as they occur *in vivo*. The proteins responsible for movement are an excellent model system for investigating such effects. In this case, the supramolecular organization is an integral component for the production of force, and thus supramolecular organization and catalysis are very closely intertwined. Additionally, the state of organization can often be manipulated experimentally so that the enzymatic properties can be determined at a range of different organizational states. This is particularly true for nonmuscle motile systems in which the structures must be capable of dynamic rearrangements such as in amoeboid movement, but it is also true of muscle, in which limited proteolysis can produce active fragments and intermediate states such as myosin minifilaments can be formed (1).

Myosin is a dimeric enzyme that catalyzes the hydrolysis of ATP and uses the free energy released by this hydrolysis to drive the mechanical contraction of muscle in conjunction with actin, the other major muscle protein (see refs. 2–5 for review). The myosin molecule is asymmetric with a long "coiled coil" tail composed of one α-helix from each monomeric unit. At the end of each helix is a globular head group, which contains the sites for ATP hydrolysis and actin binding. At physiological ionic strength, myosin molecules aggregate into filaments through alignment of their tail regions. The globular head groups project away from the filament, where they can interact with actin filaments. Limited proteolysis can cleave myosin at the neck region joining the head group and the tail to produce monomeric subfragment 1 (S1) as a single head group that retains its ATPase active site. Under other conditions, proteolysis can produce a dimeric fragment, heavy meromysin (HMM), which contains two head groups joined together by a shortened tail segment. Both HMM and S1 remain soluble at physiological ionic strength because they lack the terminal region of the tail that produces aggregation.

The kinetics of S1 are reasonably simple, unlike the kinetics of filamentous myosin, which can be complex. It is generally felt that this complexity is due at least in part to the supramolecular organization of the myosin into filaments. Sleep *et al.* (6) have proposed that the marked differences between acto-S1 and actomyosin observed in the ^{18}O-exchange patterns may be due to the multipoint attachment of myosin to actin. Such multipoint attachment is possible with a myosin filament but not with monomeric S1.

In this context, it is interesting that intermediate systems such as HMM or myosin minifilaments show fairly simple kinetics similar to those of S1 (7). Taylor (4) has presented a comprehensive review of the earlier evidence indicating a general lack of major differences between S1 and HMM in kinetics and in binding to actin. It is clear that both head groups in HMM have substantial freedom of movement relative to each other (8, 9) and that both head groups can bind to actin simultaneously (9). Both S1 and HMM have similar ATPase rates expressed on a per head basis either for the basal or actin-activated reaction. The strength of binding of HMM to actin in the presence of ADP or substrate analogs is similar to that for S1 binding to actin, while in the absence of nucleotides, the binding of HMM is up to 1000 times stronger than S1 binding (10–12).

We now report that the similarity observed between S1 and HMM in ATPase kinetics is due to the high ATP level that is usually employed. This high ATP level saturates both heads of HMM and prevents any differential interaction of the heads with actin. At lower ATP levels, striking differences are observed between the kinetics of monomeric S1 and dimeric HMM that are consistent with the existence of a tethered acto-HMM species that has one HMM head group tightly bound to actin while the other head group can turn over rapidly.

MATERIALS AND METHODS

Myosin and its subfragments were prepared from the hind legs and back of New Zealand White rabbits essentially as described by Margossian and Lowey (12). HMM was prepared by digestion of myosin with chymotrypsin in the presence of Mg^{2+} and purified by chromatography on DEAE-cellulose. S1 was prepared by hydrolysis of myosin with chymotrypsin and purified on DEAE-cellulose. The S1 was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: S1, subfragment 1; HMM, heavy meromyosin.

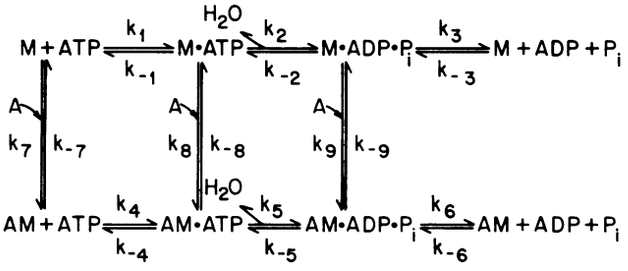
a mixture of the A1 and A2 isozymes and was enriched in A2.

All reactions were performed in 35 mM Tris·HCl/30 mM KCl/2 mM $MgCl_2$/0.1 mM EGTA/0.1 mM dithiothreitol at pH 8.2 and 25°C. The reaction mixtures also contained pyruvate kinase at 1 mg/ml and 1 mM phospho*enol*pyruvate to regenerate ATP. For measurement of ATPase rates, lactate dehydrogenase at 0.2 mg/ml and 0.3 mM NADH were included and the rate was determined from the change in absorbance at 340 nm, using an extinction coefficient of 6220 $M^{-1}\cdot cm^{-1}$ for NADH.

General methods for analysis of ^{18}O isotopic exchange were as previously reported (13–15); the ethyl derivative of phosphate was used for mass spectral analysis. [^{18}O]Phospho*enol*pyruvate was synthesized from [γ-^{18}O]GTP by using phospho*enol*pyruvate carboxykinase.

RATIONALE

Previous rapid kinetic analyses have indicated that ATP binds to acto-S1 in a multistep process with a net rate constant, k_4 of Scheme 1 (in which M represents S1, A represents actin, and AM represents acto-S1),



SCHEME 1.

of approximately 3×10^6 $M^{-1}\cdot sec^{-1}$ and that the binding of ATP induces rapid dissociation of the S1 from the actin at a rate, k_{-8}, in excess of 1000 sec^{-1}. The binding of the S1·products complex to actin is considerably weaker than the binding of free S1 to actin and consequently the S1 and actin will be predominately associated in the absence of substrate and predominately dissociated in the presence of substrate under the usual experimental conditions. Release of the products ADP and P_i in step 3 is slow but is stimulated by rebinding of the S1·products complex to actin via steps 9 and 6. With the reaction conditions employed in this study, the product release rate for S1 alone, k_3, is 0.04 sec^{-1}, while actin stimulates the ATPase rate with a value of approximately 100 μM for half-maximal stimulation and a value of approximately 20 sec^{-1} for the maximal velocity on extrapolation to infinite actin (15). We will be interested here in conditions in which actin is in molar excess over S1 or HMM head groups but is still at a low enough absolute actin concentration so that S1 or HMM will be predominately dissociated from actin in the presence of ATP and so that the ATPase rate will still be only a small fraction of the maximal rate at saturating actin. The net rate of product release indicated by k_a in Fig. 1 is the sum of the product release rate by both the basal and the actin-stimulated pathways.

With the monomer S1, the S1·products complex resulting from ATP-induced dissociation of acto-S1 has only a low stimulation of its ATPase rate because of the unfavorable diffusional barrier required for rebinding to actin. With the dimer HMM, however, as indicated in Fig. 1, the binding of one ATP induces dissociation of that one head group from the actin, but the HMM dimer remains tethered to the actin filament via the other head group, which has not bound ATP and remains strongly attached to the actin. This tethered head with product bound will see a high local actin concentration and can release product rapidly provided that it has sufficient freedom of movement to rebind to the actin and undergo the conformational changes required for actin-stimulated product release. At higher ATP levels, net dissociation of HMM from actin will occur because ATP can bind simultaneously to both heads. This dissociated HMM will experience a diffusional barrier for rebinding to actin of the same magnitude as that for S1 and any rate acceleration due to the tethered acto-HMM species will be lost.

RESULTS

ATPase Rates. Results of an experiment designed to test for an accelerated rate of ATP hydrolysis at low ATP by tethered acto-HMM are shown in Fig. 2. An ATP-regenerating system of pyruvate kinase and phospho*enol*pyruvate was used so that low ATP levels could be maintained without accumulation of ADP. The ATPase rate of acto-S1 has a simple dependence on ATP concentration with a continuous increase to a plateau value at high ATP. Acto-HMM, in contrast, has an increased ATPase rate at low ATP levels but then is inhibited by further increase in the ATP concentration. At high ATP levels the rate with acto-HMM has decreased to a value which is similar to that for acto-S1. This overshoot of the acto-HMM ATPase rate at low ATP levels

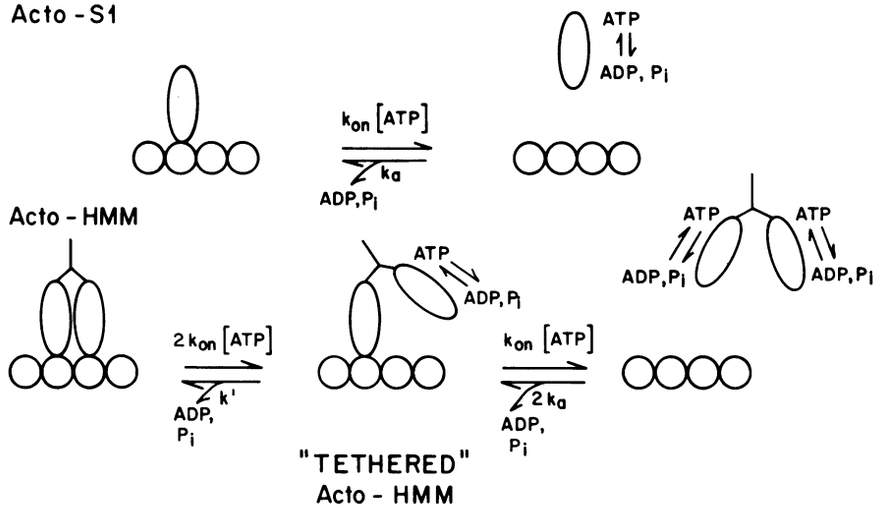


FIG. 1. Kinetic model for acto-S1 and acto-HMM. The small circles represent actin monomers in an actin filament and the larger ovals represent myosin head groups.

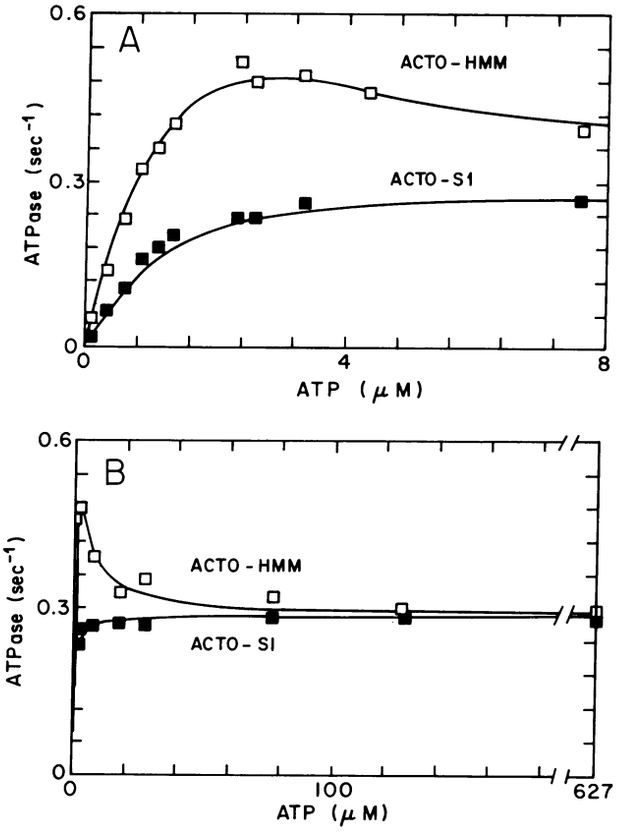


FIG. 2. Dependence of ATPase rate of acto-S1 and acto-HMM on total ATP concentration with an ATP-regenerating system. The concentrations of S1, HMM, and actin were 1.1, 1.2, and 1.9 μM, respectively. Rates are expressed on a per head group basis. The indicated ATP concentration refers to total added nucleotide. Note different abscissas in *A* and *B*.

is exactly the behavior predicted to result from formation of a tethered acto-HMM species that can release product rapidly. The lines indicated in Fig. 2 are theoretical lines calculated for the model of Fig. 1 by the King–Altman procedure (16) with correction of the free ATP level for the steady-state binding of ATP to S1 and HMM. The parameters used in the fit were $k_{on} = 0.7 \times 10^6$ $M^{-1}\cdot sec^{-1}$; $k_a = 0.283$ sec^{-1} from the observed steady-state rate for S1 at saturating ATP; and $k' = 3$ sec^{-1} as the product release rate from the tethered acto-HMM. The ATP concentration dependence of the inhibition of the ATPase rate of acto-HMM at higher ATP levels is determined by the relative values of k' and $k_{on}[ATP]_{free}$, which control the partitioning of the tethered acto-HMM intermediate.

This overshoot of the ATPase rate of acto-HMM at low ATP has been observed with four independent HMM preparations, including one prepared by hydrolysis with trypsin that lacked an intact LC2 light chain. No overshoot has been observed with a number of S1 preparations, including one prepared with papain in the presence of Mg^{2+} that contained substantial amounts of intact LC2 light chain. These results favor the interpretation that the overshoot is due to the dimeric nature of HMM and not to differences in the extent and site of proteolysis between preparations. Observation of a pronounced overshoot as seen in Fig. 2 requires a highly efficient ATP regenerating system and the proper relative and absolute concentrations of HMM and actin. Consequently it is reasonable that such an overshoot has not been previously noted.

^{18}O Oxygen Exchange. With S1 or HMM alone the reformation of bound ATP from bound ADP and P_i via reversal of step 2 of Scheme 1 is much faster than product release via step 3. Thus many cycles of hydrolysis and reformation of bound ATP occur during each turnover, with an extensive incorporation of water-derived oxygens into the P_i that is finally released (17, 18). The product release rate of S1 is so slow that nearly all four of the P_i oxygens are derived from water oxygens and the three oxygens originally attached to the γ-phosphate of ATP are lost to the water pool and do not appear in the product P_i. Binding of the S1·products complex to actin provides a faster route for product release, which both accelerates the net ATPase rate and decreases the extent of incorporation of water-derived oxygens into the P_i. This decreased oxygen exchange results from the decreased lifetime of the S1·products complex during a single turnover with a corresponding decrease in the number of reversals of the hydrolytic step that can occur before product release. This expected decrease in oxygen exchange with increasing actin stimulation has been observed at high ATP levels (6, 19, 20).

The model of Fig. 1 predicts that the rapid product release from the tethered acto-HMM intermediate, which is caused by the high local concentration of actin, should result in a decrease in the amount of oxygen exchange corresponding to that observed with S1 at high actin levels. Fig. 3 presents the results of an oxygen exchange experiment performed during steady-state ATP hydrolysis with conditions similar to those employed in the rate study of Fig. 2. In this experiment, highly ^{18}O-enriched phospho*enol*pyruvate was used to maintain the low steady-state levels of [γ-^{18}O]ATP that are required. Since the extent of activation by actin is low, the product release rate with acto-S1 is also low and a high extent of exchange occurs with acto-S1, as indicated by the extensive observed loss of ^{18}O and its replacement with ^{16}O from the unlabeled water pool. In fact with acto-S1, the pre-

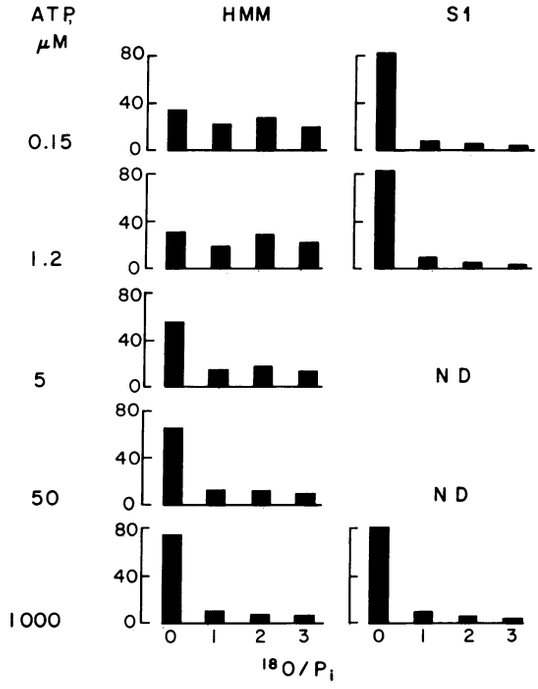


FIG. 3. ^{18}O exchange by acto-S1 and acto-HMM during hydrolysis of ATP. [^{18}O]Phospho*enol*pyruvate was used to maintain the low steady-state levels of [γ-^{18}O]ATP, which was hydrolyzed in unenriched water. The distribution of ^{18}O-labeled phospho*enol*pyruvate species was 1.2, 9.7, 36.4, and 52.7% for the species containing zero to three ^{18}O oxygens per phospho*enol*pyruvate, respectively. The concentrations of S1, HMM, and actin were 1.2, 1.2, and 2.9 μM, respectively. The reaction was initiated by addition of ADP to yield the indicated concentration of ATP. ND, not determined.

dominant species contains no ^{18}O oxygens, only four water-derived ^{16}O oxygens. This extensive oxygen exchange, which is indicative of slow product release, is observed at all ATP levels with acto-S1. With acto-HMM similar extensive exchange is observed at high ATP levels, consistent with slow product release as seen with acto-S1. At low ATP concentrations, however, acto-HMM hydrolyzes ATP with a greatly decreased level of oxygen exchange as expected for rapid product release from the tethered acto-HMM species. The increased amounts of P_i being produced at low ATP with up to three ^{18}O oxygens per P_i require that the major part of the hydrolysis must be occurring through a pathway with only approximately two reversals of the hydrolytic step per turnover as compared with an average of 30 reversals occurring during turnover at high ATP.

The fraction of ATP hydrolysis occurring by the low-exchange pathway decreases at higher ATP levels and is replaced with ATP hydrolysis by the high-exchange pathway characteristic of acto-S1. The range of ATP concentrations in which this transition between the two pathways occurs corresponds to the ATP range in which the overshoot of the acto-HMM ATPase is inhibited as predicted by the model of Fig. 1. The same pattern of oxygen exchange was observed with a different pair of independent S1 and HMM preparations (not shown).

A complication in the analysis of many preparations of myosin and its subfragments is the presence of a variable amount of ATP hydrolysis with a low extent of oxygen exchange (21, 22). This low-exchange pathway that is observed at high ATP levels is not well understood and may be due to multiple causes with different preparations and experimental conditions. We have reported evidence that this low-exchange component with acto-HMM is due to a small fraction of the HMM that is maximally stimulated at low actin levels (15). This variable amount of a low-exchange pathway at high ATP levels appears to represent a separate phenomenon from the low-exchange pathway observed in Fig. 3 at low ATP levels due to the participation of tethered acto-HMM.

DISCUSSION

The evidence presented here indicates that tethered acto-HMM does exist at low ATP concentrations and that it can turn over rapidly due to the high effective actin concentration that it experiences. This model can account for the increased ATPase rate at low ATP of acto-HMM relative to acto-S1 and the inhibition of this extra ATPase rate at higher ATP levels as seen in Fig. 2. The oxygen exchange experiments of Fig. 3 provide striking evidence for a major increase in the rate of release of product relative to reformation of bound ATP by acto-HMM at low ATP levels, but not at high ATP levels or with acto-S1 at either low or high ATP level. Direct binding studies have also recently indicated that HMM can bind to actin with only one head in the presence of ADP (23). The kinetic properties of the tethered acto-HMM are analogous in many aspects to those of the chemically crosslinked acto-S1 complex (24). Interestingly, recent observations with the dynein–tubulin system have also indicated a requirement for ATP binding to all three dynein heads for the net dissociation of dynein from tubulin (25).

The model of Fig. 1 provides an understanding of why little effect on the ATPase rate at high ATP is observed for the conversion of the monomer S1 into the dimer HMM. At high ATP the tethered acto-HMM species is still transitorily formed, via the reverse of the second step of the scheme for acto-HMM in Fig. 1, but the ATP binding to the head that is bound to actin is so fast that net dissociation of the HMM from the actin usually occurs before significant product release from the tethered head via k'. Acto-HMM at high ATP in effect operates with one head at a time because $k' << k_{on}[ATP]_{free}$.

Other lines of evidence, especially with nonskeletal myosins (26, 27), have indicated cooperative interactions between the head groups of HMM and myosin. There is no need to propose any special interaction between the two heads of HMM in order to account for the data presented here with HMM from skeletal muscle. The complex kinetics of acto-HMM at low ATP can be adequately accounted for by the simple model of Fig. 1, using the rate parameters of acto-S1 with the addition of k', which is unique to acto-HMM. Such cooperative interactions are not excluded, however, if indicated by additional experiments.

The simple model of Fig. 1 assumes that the only change between the rate of HMM and S1 rebinding to actin is the statistical factor of 2. This seems to be a reasonable approximation for acto-HMM, but is not likely to remain valid for larger organized structures such as myosin filaments. It has been appreciated for some time that many of the unusual kinetic properties of filamentous myosin are related to the organization of the system into such a complex supramolecular structure that has to interact with a second filamentous protein in actin. The results with acto-HMM reported here provide a quantitative understanding of this relatively simple system and will constitute the foundation for the development of a more meaningful, quantitative appreciation for the influence of supramolecular organization on the actomyosin ATPase.

The rate for k' of 3 sec^{-1} used to fit the ATPase rate data is fast, but is still less than the maximal rate of 20 sec^{-1} at saturating actin levels. This decreased value for k' is also supported by the oxygen exchange measurements. Although the exchange is markedly reduced with tethered acto-HMM, there is still significant exchange. With acto-S1 at high ATP, in contrast, there is little or no residual exchange at saturating actin (20). Thus the persistence of some exchange with tethered acto-HMM indicates that the effective actin concentration is not saturating.

Several factors may contribute to this reduced value of k' relative to the maximal velocity and the reduced value of k_{on} relative to the expected ATP binding rate. The model of Scheme 1 and Fig. 1 is certainly an oversimplification and the indicated steps are known to be complex, with multiple kinetic intermediates between the principal indicated species. For example, it is known that actin stimulates release of ATP as well as release of ADP and P_i from the steady-state mixture of bound ATP and bound ADP and P_i (28). At high ATP, this ATP release does not inhibit the overall rate as ATP rebinding will be fast, but at low ATP levels, rebinding will be slow and the net rate will be reduced. This factor could not, however, account for the persistence of oxygen exchange by tethered acto-HMM.

Another factor that is likely to contribute to the decreased value of k' relates to the degree of freedom of the tethered acto-HMM head after release from the actin. Although the tethered head will not have a gross translational diffusion barrier for rebinding to actin, it still will have some freedom to move about the flexible region joining it to the rest of the HMM molecule. The requirement for the loss of this freedom on rebinding to actin will result in a lower observed rate relative to the extrapolated value at infinite actin (e.g., since the tethered HMM head can partially move away from actin, the effective local actin concentration will not be infinite). In addition to this kind of entropic effect, rebinding of the tethered head may also be disfavored by enthalpic factors. The range of orientations that are required for rebinding to actin and the conformational changes accompanying product release may not be the most favorable orientations for the tethered head group, given the orientational restraints imposed by the linkage to the other head group. Any strain imposed

by such unfavorable orientations will reduce the observed value of k'. Such partial hindrance to simultaneous binding of both heads to actin has been postulated to be the cause of the lack of a large increase in the strength of HMM binding to actin over the strength of S1 binding (10).

This work was supported in part by Grant AM25980 from the U.S. Public Health Service and was performed during the tenure of D.D.H. as an Established Investigator of the American Heart Association.

1. Reisler, E., Smith, C. & Seegan, G. (1980) *J. Mol. Biol.* **143,** 129–145.
2. Trentham, D. R., Eccleston, J. F. & Bagshaw, C. R. (1976) *Quart. Rev. Biophys.* **9,** 217–281.
3. Lymn, R. W. (1979) *Annu. Rev. Biophys. Bioeng.* **8,** 145–163.
4. Taylor, E. W. (1979) *CRC Crit. Rev. Biochem.* **6,** 103–164.
5. Adelstein, R. S. & Eisenberg, E. (1980) *Annu. Rev. Biochem.* **49,** 921–956.
6. Sleep, J. A., Hackney, D. D. & Boyer, P. D. (1980) *J. Biol. Chem.* **255,** 4094–4099.
7. Reisler, E. (1980) *J. Biol. Chem.* **255,** 9541–9544.
8. Mendelson, R. A., Morales, M. F. & Bitts, J. (1973) *Biochemistry* **12,** 2250–2255.
9. Thomas, D. D., Seidel, J. C., Hyde, J. S. & Gergely, J. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1729–1733.
10. Highsmith, S. (1978) *Biochemistry* **17,** 22–26.
11. Greene, L. E. (1981) *Biochemistry* **20,** 2120–2126.
12. Margossian, S. S. & Lowey, S. (1982) *Methods Enzymol.* **85,** 55–71.
13. Hackney, D. D., Stemple, K. E. & Boyer, P. D. (1980) *Methods Enzymol.* **64,** 60–83.
14. Hackney, D. D. (1980) *J. Biol. Chem.* **255,** 5320–5328.
15. Hackney, D. D. (1982) *J. Biol. Chem.* **257,** 9494–9500.
16. King, E. L. & Altman, C. (1956) *J. Phys. Chem.* **60,** 1375–1378.
17. Bagshaw, C. R., Trentham, D. R., Wolcott, R. G. & Boyer, P. D. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 2592–2596.
18. Webb, M. R. & Trentham, D. R. (1981) *J. Biol. Chem.* **256,** 10910–10916.
19. Shukla, K. K. & Levy, H. M. (1977) *Biochemistry* **16,** 132–136.
20. Sleep, J. A. & Boyer, P. D. (1978) *Biochemistry* **17,** 5417–5422.
21. Shukla, K. K., Levy, H. M., Ramirez, F., Marecek, J. F., Meyerson, S. & Smith, E. S. (1980) *J. Biol. Chem.* **255,** 11344–11350.
22. Midelfort, C. F. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2067–2071.
23. Manuck, B. A., Seidel, J. C. & Gergely, J. (1984) *Biophys. J.* **45,** 222a (abstr.).
24. Mornet, D., Bertrand, R., Pantel, P., Audemard, E. & Kassab, R. (1981) *Nature (London)* **292,** 301–306.
25. Shimizu, T. & Johnson, K. A. (1983) *J. Biol. Chem.* **258,** 13841–13846.
26. Persechini, A. & Hartshorne, D. J. (1981) *Science* **213,** 1383–1385.
27. Chantler, P. D., Sellers, J. R. & Szent-Gyorgyi, A. G. (1981) *Biochemistry* **20,** 210–219.
28. Sleep, J. A. & Hutton, R. L. (1978) *Biochemistry* **17,** 5423–5430.