Intermediate Oxygen Exchange Catalyzed by the Actin-Activated Skeletal Myosin Adenosinetriphosphatase†

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ABSTRACT: Considerable effort has been devoted to understanding the mechanism of ^{18}O exchange in skinned skeletal and insect muscle fibers. However, a full understanding of the mechanism of ^{18}O exchange in muscle fibers requires an understanding of the mechanism of ^{18}O exchange in the simpler in vitro systems employing myosin subfragment 1 (S-1) and heavy meromyosin (HMM). In the present study, using both S-1 and S-1 covalently cross-linked to actin, we show first that over a wide range of temperature, ionic strength, and actin concentration there is only one pathway of ^{18}O exchange with S-1. This is also the case with HMM except at very low ionic strength and low actin concentration, and even here, the data can be explained if 20% of the HMM is denatured in such a way that it shows no ^{18}O exchange. Our results also show that actin markedly decreases the rate of ^{18}O exchange. If it is assumed that P_i release is rate limiting, the four-state kinetic model of the actomyosin ATPase cannot fit these ^{18}O exchange data. However, if it is assumed that the ATP hydrolysis step is rate limiting and P_i release is very fast, the four-state kinetic model can qualitatively fit these data although the fit is not perfect. A better fit to the ^{18}O exchange data can be obtained with the six-state kinetic model of the actomyosin ATPase, but this fit requires the assumption that, at saturating actin concentration, the rate of P_i rotation is about 9-fold slower than the rate of reversal of the ATP hydrolysis step.

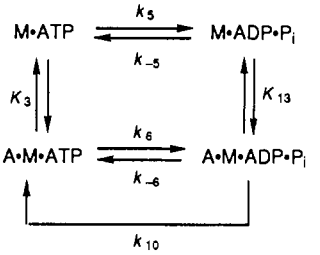
During the last several years, there have been a number of studies on ^{18}O exchange in the actomyosin system. These studies have been carried out both in solution and in skinned muscle fibers (Sleep et al., 1980; Hibberd et al., 1985; Lund et al., 1988; Shukla et al., 1988). Earlier work on ^{18}O exchange with myosin in the absence of actin showed that a large amount of ^{18}O exchange occurs in this system because of the rate of certain key steps in the myosin ATPase cycle (Levy & Koshland, 1959; Sartorelli et al., 1966). The relevant steps in an ATPase cycle which determine the amount of ^{18}O exchange are shown in Scheme I where E is any enzyme.

Scheme I

$$E\cdot ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot ADP\cdot P_i \xrightarrow{k_2} E\cdot ADP + P_i$$

Bagshaw and Trentham (1974) demonstrated that a large amount of ^{18}O exchange occurs as myosin hydrolyzes ATP because the reverse transition from M·ADP·P_i to M·ATP, k_{-1}, is much faster than k_2, the rate of P_i release from M·ADP·P_i, which is the rate-limiting step in the myosin ATPase cycle and, in addition, the binding of ATP is essentially irreversible (Bagshaw et al., 1974). Therefore, during each cycle of ATP hydrolysis, the back-transition from M·ADP·P_i to M·ATP, k_{-1}, occurs many times before P_i is released into solution. Each one of these reversals causes the P_i molecule to lose an oxygen atom, and then, when hydrolysis reoccurs, this oxygen atom is replaced by an oxygen atom from a water molecule. If the P_i molecule rotates in the active site of the myosin during this process, all of the oxygens of the P_i molecule will gradually be replaced by water oxygens, a process which can be monitored by labeling the phosphoryl group with ^{18}O.

Scheme II: Four-State Model[a]



[a] A = myosin; M = myosin subfragment 1.

Although the process of ^{18}O exchange with myosin in the absence of actin is well understood in terms of the rate constants in the myosin ATPase cycle, a similar understanding has not been developed for the actomyosin ATPase cycle. The most detailed studies on ^{18}O exchange in the presence of actin have been carried out using the soluble proteolytic fragments of myosin, S-1 and HMM. One observation made clear by these studies is that the extent of ^{18}O exchange markedly decreases when actin is present (Sleep & Boyer, 1978). This is most likely due to the ability of actin to activate the myosin ATPase activity or, more specifically, to increase the rate of P_i release from myosin. Obviously, if P_i release is more rapid, there will be less chance for reversals of the ATP hydrolysis step to occur, and, therefore, there will be less ^{18}O exchange.

However, there are difficulties with this simple model. First, the amount of ^{18}O exchange depends not only on the rate of breakdown of A·M·ADP·P_i but also on the rate of the reverse transition from A·M·ADP·P_i back to A·M·ATP. Quantitatively, by use of the rate constants in Scheme I, this means that the amount of ^{18}O exchange depends on the partition coefficient (P_c), the ratio $k_{-1}/(k_{-1} + k_2)$ (Hackney, 1980). Unfortunately, there is disagreement about the values of these rate constants in the presence of actin. Both a four-state model

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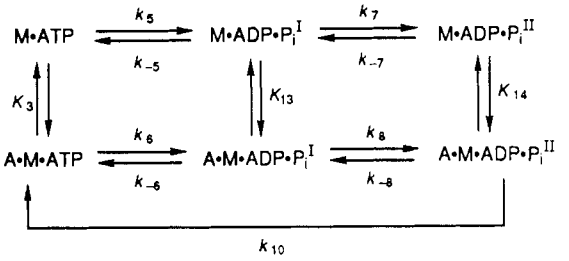
Scheme III: Six-State Model[a]



[a] A = myosin; M = myosin subfragment 1.

(Scheme II) and a six-state kinetic model (Scheme III) for the actomyosin ATPase activity have been proposed which differ in the nature of the rate-limiting step (Rosenfeld & Taylor, 1984; Stein et al., 1984). In the four-state model, either the ATP hydrolysis step or the P_i release step could be rate limiting while in the six-state model, a special step following the ATP hydrolysis step has been postulated to be rate limiting. Therefore, depending on the model and the choice of rate constants, there is considerable variation in the predicted amount of ^{18}O exchange.

For example, in the four-state model, if k_{10}, the rate of P_i release, were the rate-limiting step in the actomyosin ATPase cycle as it is in the myosin ATPase cycle, there would be considerable ^{18}O exchange because the reverse rates k_{-5} and k_{-6} are, in fact, relatively fast compared to the overall rate of the actomyosin ATPase activity. This would also be the case if the six-state kinetic model of the actomyosin ATPase activity were valid. Although, a special rate-limiting step rather than the P_i release step is rate limiting in this model, the rate-limiting step follows the ATP hydrolysis step, and, therefore, in respect to ^{18}O exchange, the model acts nearly identically with a four-state model where P_i release is rate limiting. On the other hand, if the ATP hydrolysis step were rate limiting and was followed by rapid release of P_i, which is another possibility in the four-state kinetic model, then considerably less ^{18}O exchange would be predicted.

Another difficulty with understanding ^{18}O exchange in the actomyosin system is that there have been persistent reports of more than one pathway of ^{18}O exchange occurring when the two-headed proteolytic fragment of myosin, HMM, is used; it has been suggested that this is due to differences in the ATPase cycle carried out by the two myosin heads (Shukla et al., 1988; Levy & Koshland, 1959; Midelfort, 1981). Studies on ^{18}O exchange in skinned muscle fibers or with precipitated actomyosin have also shown that at least two pathways of ^{18}O exchange occur in these systems. In addition, the amount of ^{18}O exchange which occurs in these systems is very different from that which occurs with the soluble systems, acto·S-1 and acto·HMM. The nature of these differences is not understood at the present time (Hibberd et al., 1985; Hackney, 1982; Ikeuchi & Midelfort, 1986).

In the present study, we carried out a detailed study of ^{18}O exchange with both S-1 and HMM. We also worked with cross-linked acto·S-1 over a wide range of temperature and ionic strength. Our results suggest, first, that in almost all cases there is only one pathway of ^{18}O exchange and in the one case where this is not the case, it could well be due to denatured HMM. Second, our results show that, while actin markedly decreases the rate of ^{18}O exchange, this either could be due to a very fast rate of P_i release consistent with the four-state kinetic model of the actomyosin ATPase activity or could be consistent with the six-state kinetic model of the actomyosin ATPase provided that at saturating actin concentration the rate of P_i rotation is about 9-fold slower than the rate of reversal of the ATP hydrolysis step.

Materials and Methods

Proteins. Actin and S-1 were prepared as described by Stein et al. (1979). The (A1)S-1 and (A2)S-1 isozymes were prepared as given in Chalovich et al. (1984). Protein concentrations were determined by ultraviolet spectroscopy using molecular weights of 42000 and 120000, respectively, for actin and S-1 (Stein et al., 1979). Cross-linked actin·S-1 was prepared by the Stein et al. (1985) modification of the method of Mornet et al. (1981). A trace of ^{14}C-labeled S-1 was included to facilitate quantitation of the cross-linked proteins. Tryptic HMM was prepared by the method of Margossian and Lowey (1982). Chymotryptic HMM was prepared either by the method of Margossian and Lowey (1982) as modified by Shukla et al. (1988), which includes 2 mM $CaCl_2$ during digestion, or by the method of Okamoto and Sekine (1985), without $CaCl_2$.

[^{18}O]ATP. ATP labeled in the γ-phosphoryl group was synthesized by a modification of the method of von der Saal et al. (1985) using a ratio of 2 mol of phosphate/mol of ADP. The extent of reaction was monitored by HPLC using a 25 cm × 4.1 mm SynchroPak AX-300 ion-exchange column (SynChrom, Inc., Linden, IN). The isotopic purity was assayed by monitoring the chemical shift imparted to a ^{31}P nucleus when bound to an ^{18}O nucleus (Cohn & Hu, 1980). The NMR spectra were obtained at 121.5 MHz on a Bruker WM-300 instrument (Bruker Instruments, Inc., Billerica, MA). We thank Dr. William Egan of the Food and Drug Administration for performing the analyses. The ATP was purified by chromatography through DEAE-Sephadex A25-120 by elution with a 0.1–0.6 M gradient in triethylammonium bicarbonate (Yount et al., 1971; von der Saal et al., 1985). The ATP was concentrated by rotary evaporation and stored at −80 °F, either as the dry sodium salt after conversion with NaI (Yount et al., 1971) or as a methanolic solution of the triethylammonium salt (Midelfort & Rose, 1976). Both preparations gave similar results. Storage as the triethylammonium salt afforded higher percentage yields.

Chemicals and Reagents. Water, 98 atom % ^{18}O, was obtained from Amersham (Arlington Heights, IL). Phosphorus pentachloride and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were from Aldrich (Milwaukee, WI). Anion-exchange resins (AG-1 X8) were from Bio-Rad (Richmond, CA). Potassium cyanate was from Alfa/Thiokol (Danvers, MA). HPLC grades of diethyl ether, chloroform, methanol, and acetone were from Burdick and Jackson (Muskegon, MI). Li_3ADP was from P-L/Pharmacia (Piscataway, NJ). All other chemicals and reagents were from Sigma (St. Louis, MO).

Oxygen Exchange. Equilibrium oxygen exchange accompanying ATP hydrolysis was determined by isolation of the liberated phosphate and conversion to the trimethyl derivative (Hackney et al., 1980). The distribution of isotopic oxygen phosphate species was determined by combined gas chromatography/mass spectroscopy on a Finnigan Model 4021 instrument (Finnigan MAT, San Jose, CA) using chemical ionization. Monitoring the mass spectrum at mass/charge (m/e) ratios of 141, 143, 145, 147, and 149 reflects phosphate with respectively one to four atoms of ^{18}O per molecule. We thank Robert Smith of the University of West Virginia Department of Biochemistry Mass Spectroscopy Center for performing these assays. The oxygen isotopic composition was used to calculate a partition coefficient (P_c) and a theoretical distribution assuming a single path using algorithms written in MLAB (Knott, 1979) or BASIC. We thank Dr. David D.

Hackney for his gift of a diskette containing BASIC routines from which these algorithms were derived.

Phosphate Contamination. Hydrolysis of ATP by myosin in the presence of calcium ions results in the incorporation of one atom of oxygen derived from solvent water into the product phosphate. Apart from the one obligate oxygen, no further solvent-derived oxygens are incorporated into the phosphate; i.e., there is no intermediate phosphate–water exchange activity under these conditions (Swanson & Yount, 1966). This property has been exploited by many experimenters to establish the oxygen isotopic composition of the γ-phosphoryl of the substrate ATP. In our experiments, each observation is made by using 4 μmol of ATP quantitatively hydrolyzed as monitored by pH-stat titration. After conversion to the trimethyl ester, 1–2 nmol is assayed by GC–MS as described above. We have observed random interexperimental variation in the distribution of isotopic phosphate species yielded by a single preparation of [^{18}O]ATP hydrolyzed by S-1 in the presence of Ca ions. The variation is only a few percent of the total phosphate in the sample and represents an excess of the naturally abundant species [$^{16}O_4$]P_i. Cleaning the experimental glassware in 0.1 N HCl tends to suppress but not eliminate the variability in the observed distribution. Under conditions such that ATP hydrolysis is accompanied by moderate exchange activity, the slight variation in the distribution produced by the CaATPase activity is inconsequential. However, when the amplitude of the exchange process is very small, as is the case for the cross-linked actin–S-1 complex, the presence of even a small percentage of contaminating exogenous phosphate (which appears to be completely exchanged phosphate) results in a 2–3-fold increase in the apparent P_c as well as in a large experiment to experiment variation in the apparent P_c. The phosphate species containing four atoms of ^{16}O (141 atomic mass units after trimethyl esterification and chemical ionization) is a mixture with contributions from three pools: the original ATP, any due to enzymatic exchange processes, and any due to contamination by exogenous phosphate. Only those experiments involving cross-linked actin–S-1 (Figure 5) were corrected for exogenous phosphate by assuming that the predicted values for [$^{16}O_4$]P_i were correct and that the slight excess observed was due to contamination. Subtraction of this excess, renormalization to 100%, and recalculation of the P_c and predicted distributions yielded values for [$^{16}O_3$]P_i, [$^{16}O_2$]P_i, and [$^{16}O_1$]P_i that were essentially unchanged from the original distributions, but the scatter in the P_c was reduced. For example, for cross-linked actin–S-1 at 5 °C, 13 mM ionic strength, the uncorrected values of P_c were 0.07, 0.14, and 0.22, whereas after correction these values were 0.12, 0.14, and 0.16. Note that our measurements concerning the heterogeneity of HMM and the kinetic mechanism of actin activation were not corrected in this manner because the hydrolytic activity was accompanied by moderate to high amounts of exchange.

ATPase Activity. Steady-state rates were determined by using an automatic pH-stat (Eisenberg & Moos, 1967).

Results

It is not possible to analyze the results of ^{18}O exchange experiments in terms of kinetic models and rate constants unless it is clear that there is only one pathway of ^{18}O exchange. This can be tested by measuring the distribution of ^{18}O in the P_i produced during the ATPase reaction. For a given overall level of ^{18}O exchange, the percentage of P_i molecules with one, two, three, and four ^{18}O atoms is theoretically determined if there is only one pathway of ^{18}O exchange (Hackney, 1980). Figure 1 shows the distribution of

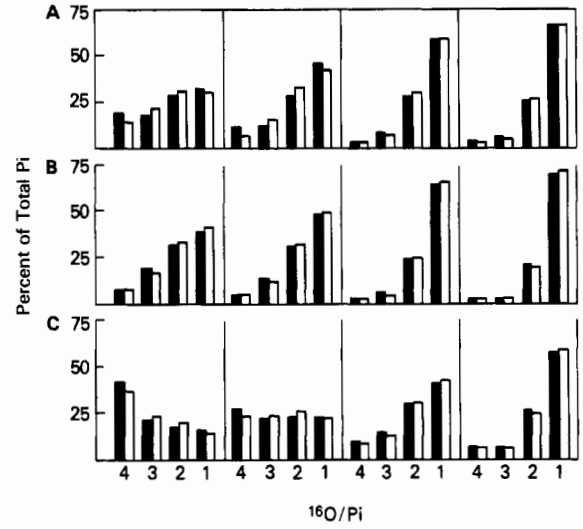


FIGURE 1: Distribution of P_i species: actin concentration-dependence. S-1 (0.1–0.4 μM) was incubated in a 13 mM ionic strength buffer containing (in mM) 2 imidazole, 1 [^{18}O]ATP, 1 DTT, 4.5 KCl, and the indicated concentration of actin in a beaker thermostated to 15 °C. The ATPase rate was determined by pH-stat titration at pH 7.0. Upon completion of the reaction, the P_i was isolated, and the distribution of oxygen isotopes was determined as described under Materials and Methods. The solid bars represent the observed percentage of phosphate molecules containing from four to one atom of oxygens from water. These percentages were summed to yield an average number of water oxygens per phosphate which is related to P_c as indicated under Materials and Methods. The open bars are the theoretical distributions assuming a single path of hydrolysis. (A) Unfractionated S1; (B) (A1)S-1; (C) (A2)S-1. From left to right, 2, 5, 20, and 100 μM actin.

P_i species produced both by unfractionated S-1 and by the two isoenzymes of S-1 which contain either the A1 or the A2 light chain. Over a range of actin concentrations from 1 to 100 μM, only one pathway of exchange is observed. Below 2 μM actin, the theoretical distribution was calculated by taking into account that a small fraction of the total phosphate is produced by S-1 which is not activated by actin. This fraction was determined by comparing the measured ATPase activity with the ATPase activity of S-1 alone.

We also investigated whether a single pathway of ^{18}O exchange occurs with S-1 covalently cross-linked to actin. This is equivalent to measuring the ^{18}O exchange at infinite actin concentration. Figure 2 shows the distribution of P_i species produced by cross-linked actin–S-1 over a wide range of temperature and KCl concentration. Under all of the conditions studied, only one pathway of exchange is observed. Preliminary experiments in 2.0 M KCl (not shown) suggested some heterogeneity. However, these conditions are so far removed from physiological that further investigation did not seem useful. Therefore, in agreement with the results of other workers, we find that only one pathway of ^{18}O exchange occurs with acto·S-1.

We next investigated whether more than one pathway of exchange occurs with acto·HMM where previous work has suggested that two pathways of exchange may occur. Since it has been reported that the occurrence of two pathways depends on the method of preparation of HMM, we tested both tryptic and chymotryptic HMM prepared in the presence or absence of 2 mM $CaCl_2$. The experiments were performed at both 13 and 50 mM ionic strength (Figure 3). Our results show that only at the lower ionic strength at actin concentrations below 10 μM do we observe any heterogeneity in the distribution if we model the data on the basis of a single pathway of exchange (Figure 3a, open bars). We observe, consistent with Shukla et al. (1988), that, in the presence of

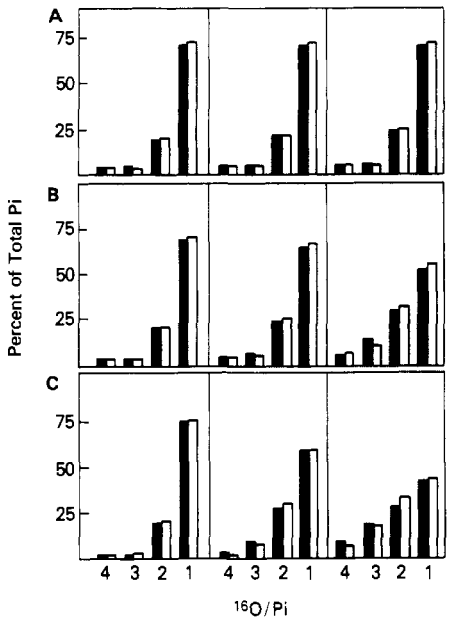


FIGURE 2: Distribution of P_i species: cross-linked actin·S-1. Cross-linked actin·S-1 (0.1 μM in S-1) was analyzed as given in Figure 1. The ionic strength was varied by the addition of KCl as appropriate. (A) 35 °C; (B) 15 °C; (C) 5 °C. From left to right, 13, 150, and 500 mM ionic strength.

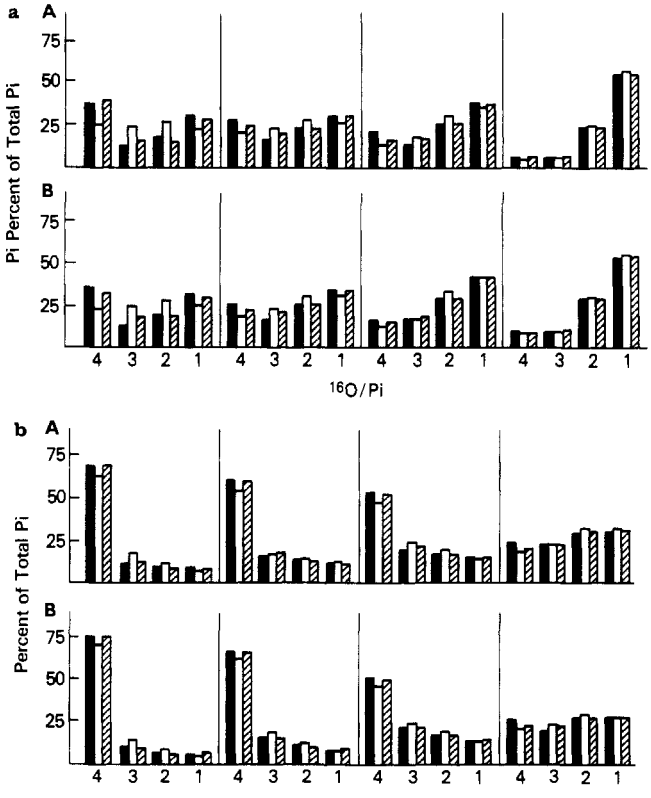


FIGURE 3: Distribution of P_i species: acto·HMM. Tryptic (top panels) or chymotryptic HMM prepared in the presence of 2 mM $CaCl_2$ (bottom panels) was analyzed as in Figure 1 at 13 mM (a) ionic strength or by the addition of KCl to 50 mM ionic strength (b). Observed (solid bars); single pathway of exchange (open bars); two pathways of exchange, 80% showing the same amount of exchange as S-1 would at the measured ATPase rate and 20% showing no exchange (hatched bars).

added KCl, both tryptic HMM and chymotryptic HMM yield homogeneous distributions (Figure 3b). Where the heterogeneity does occur, it seems to be present with both tryptic and chymotryptic HMM prepared either in the presence or in the absence of $CaCl_2$. We were able to model this heterogeneity by assuming two pathways of exchange, with 80% of the HMM showing exchange identical with that of actin-activated S-1 with the observed ATPase rate and 20% showing no exchange at all (Figure 3, hatched bars). Since it is very difficult to rule out that 20% of the HMM is denatured in some way, we do not think that this effect warrants a speculative interpretation in terms of the mechanism of the actomyosin ATPase activity.

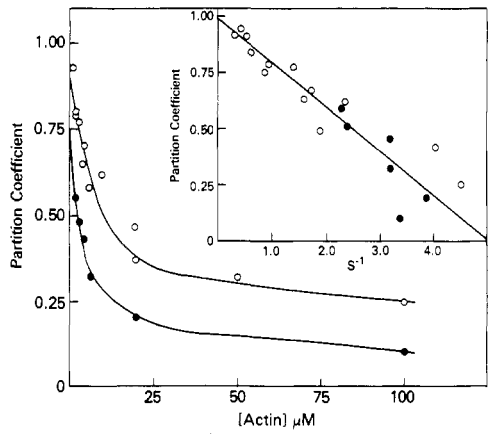


FIGURE 4: (A1)S-1 and (A2)S-1 dependence of P_c on actin concentration. (A1)S-1 (solid points) and (A2)S-1 (open points) were analyzed as in Figure 2. The inset gives the P_c as a function of the observed ATPase rate.

It should be noted that we cannot rule out that 20% denaturation is also present under the other conditions where we examined ^{18}O exchange by acto·HMM. Here too, we were able to fit the data in terms of the two pathways discussed above (Figure 3 hatched bars), but the important point is that it is not necessary to analyze the data in this manner. In general, it may only be useful to analyze the data in terms of two pathways when analyzing it in terms of one pathway is not sufficient. If this limitation is not followed, it is possible to overinterpret ^{18}O exchange data.

Having determined that, in general, myosin shows one pathway of exchange, we next investigated how the magnitude of the ^{18}O exchange depends on actin concentration and on the conditions of the measurement. Figure 4 shows the dependence of ^{18}O exchange on actin concentration with (A1)S-1 and (A2)S-1. In both cases, ^{18}O exchange decreases with an increase in actin concentration, but the decrease is significantly larger with (A1)S-1 than with (A2)S-1. Since, at a given actin concentration, (A1)S-1 has a higher ATPase activity than (A2)S-1 (Chalovich et al., 1984), we speculated that the difference in ^{18}O exchange was related to the level of the actin-activated ATPase activity. The inset in Figure 4 shows that this indeed appears to be the case; when normalized to ATPase activity, (A1)S-1 and (A2)S-1 show the same level of ^{18}O exchange. Therefore, the difference in ^{18}O exchange observed with the two isozymes probably reflects the difference in the level of actin activation which occurs with these two isoenzymes at a given actin concentration.

The observation that the level of ^{18}O exchange markedly decreases as the actin-activated ATPase rate increases suggests that there may always be a simple inverse relationship between the level of ^{18}O exchange and the level of the actin-activated ATPase activity. However, experiments on the temperature and KCl dependence of the ^{18}O exchange observed with S-1 cross-linked to actin show that this simple relationship does not always hold. The ATPase activity of cross-linked acto·S-1 increases with both an increase in temperature and an increase in ionic strength. However, Figure 5 shows that, whereas an increase in temperature does indeed decrease the level of ^{18}O

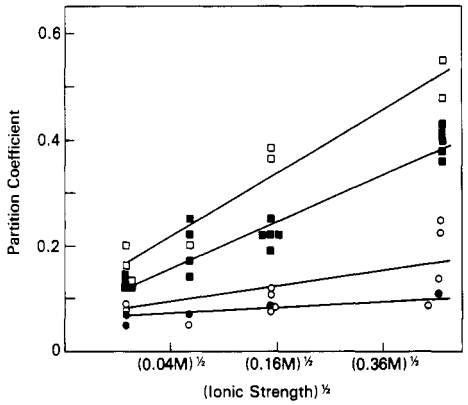


FIGURE 5: Cross-linked actin·S-1: P_c versus ionic strength. Conditions same as Figure 2.

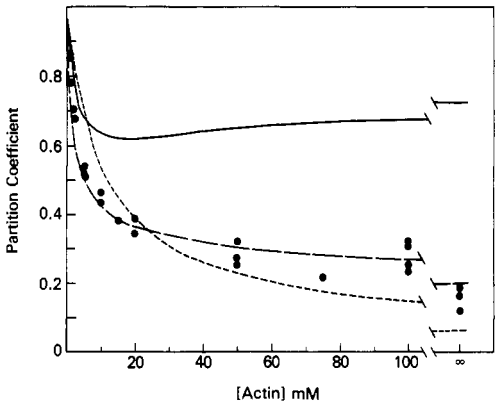


FIGURE 6: Fit to four- and six-state models. P_c values determined as in Figure 1 for unfractionated S-1 were fit to the four-state model (---): $k_5 = 21\ s^{-1}$; $k_{-5} = 7\ s^{-1}$; $k_6, k_{-6} = 3\ s^{-1}$; $k_{10} = 45\ s^{-1}$; $K_3 = 0.04165\ \mu M^{-1}$; $K_{13} = 0.0139\ \mu M^{-1}$, P_c the six-state kinetic model assuming free rotation of the P_i (—): $k_5 = 21\ s^{-1}$; $k_{-5} = 7\ s^{-1}$; $k_6, k_{-6} = 27\ s^{-1}$; $k_7, k_8 = 12\ s^{-1}$; $k_{-7}, k_{-8} = 66\ s^{-1}$; $k_{10} = 400\ s^{-1}$; $K_3 = 0.0555\ \mu M^{-1}$; $K_{13}, K_{14} = 0.0185\ \mu M^{-1}$. Value fit to a six-state model assuming a partial restriction equal to $3\ s^{-1}$ on the rate of P_i rotation along the lower path (– –): $k_5 = 18\ s^{-1}$; $k_{-5} = 6\ s^{-1}$; $k_6, k_{-6} = 27\ s^{-1}$; $k_7, k_8 = 12\ s^{-1}$; $k_{-7}, k_{-8} = 48\ s^{-1}$; $k_{10} = 500\ s^{-1}$; $K_3 = 0.0555\ \mu M^{-1}$; $K_{13}, K_{14} = 0.0185\ \mu M^{-1}$. The points at "infinite" actin concentration were obtained as in Figure 2 using cross-linked actin·S^{-1}.

exchange, ^{18}O exchange with cross-linked acto·S-1 actually increases with an increase in ionic strength. Although this could be an effect which only occurs with cross-linked acto–S-1, previous ATPase data obtained with cross-linked acto–S1 suggest it acts very similarly to S-1 at saturating actin concentration (Stein et al., 1985). In any event, the data in Figure 5 demonstrate that there is not always a simple inverse relationship between the level of the actin-activated ATPase activity and the amount of ^{18}O exchange. As we noted above, the ^{18}O exchange does not just depend on the rate of P_i release but also on the back-rate of the ATP hydrolysis step and on the rate of oxygen randomization. It is quite possible that, as the ionic strength is increased, the back-rate of the ATP hydrolysis step or the rate of P_i rotation increases more than the rate of P_i release, which may explain why both the ATPase rate and the level of ^{18}O exchange increase as the ionic strength is increased.

We next quantitatively analyzed the dependence of the ^{18}O exchange on the actin concentration in terms of the four-state and six-state models. Both previous work from our laboratory and the work of Rosenfeld and Taylor (1984) have shown that, for the four-state model to be valid at low ionic strength, the ATP hydrolysis step must be rate limiting and the subsequent rate of P_i release must be fast. We therefore modeled the ^{18}O exchange data with the assumption that the rate of the ATP hydrolysis step is $6\ s^{-1}$, the back-rate of the ATP hydrolysis step is $10\ s^{-1}$, and the rate of P_i release is $50\ s^{-1}$. This set of rate constants fits the ATPase data about as well as we have shown in previous publications. The fit to the ^{18}O exchange is shown in Figure 6. As can be seen, the fit is reasonable but not perfect, with the level of ^{18}O exchange dropping more rapidly as a function of actin concentration than is predicted by the theoretical plot.

We next tested whether the six-state model could fit these data. In the six-state model, the rate-limiting step in the cycle follows the ATP hydrolysis step which is postulated to be rapid. In this regard, the model is very similar to the four-state model with the P_i release step rate limiting. The difference in the two models is that the six-state model can explain the difference between K_{ATPase} and $K_{binding}$ which is observed at low ionic strength but the four-state model with the P_i release step rate limiting cannot explain these data since it predicts that K_{ATPase} and $K_{binding}$ will be nearly equal (Stein et al., 1984). When we fit our data to the six-state model, we found that it predicted much more ^{18}O exchange than was actually observed as the actin concentration increased. This is not surprising since this model has a rather rapid back-rate for the ATP hydrolysis step, but the rate-limiting step which follows the ATP hydrolysis step is rather slow. Therefore, it is clear that neither the six-state model nor the four-state model with P_i release rate limiting can fit the ^{18}O exchange data. Both predict too much exchange.

We next tested whether a limitation in the rate of P_i rotation at the active site would be sufficient to fit the data to the six-state model. Generally, it is assumed that the rate of P_i rotation at the active site is much faster than the back-rate of the ATP hydrolysis step. The long-dashed line in Figure 6 shows that making the rate of P_i rotation $3\ s^{-1}$ compared to $27\ s^{-1}$ for the back-rate of the ATP hydrolysis step is sufficient to make the six-state model fit the ^{18}O exchange data almost perfectly.

Discussion

Considerable effort is presently under way to understand the mechanism of ^{18}O exchange in skinned skeletal and insect muscle fibers (White et al., 1987). However, a full understanding of the mechanism of exchange in fibers will undoubtedly require an understanding of the mechanism of ^{18}O exchange in the much simpler in vitro systems, acto·S-1 and acto·HMM.

In the present study, we had to first determine whether S-1 and HMM show a single pathway of ^{18}O exchange in the presence of actin. Our results show that, except at very low ionic strength at low actin concentration, the data can be reasonably fit by assuming one pathway of exchange. Even with HMM at low ionic strength and low actin concentration, it is possible to fit the data assuming that 80% of the HMM shows exchange identical with S-1 and 20% of the HMM shows no exchange at all. Given the observation that myosin preparations show heterogeneity with regard to the irreversible binding of ATP and the magnitude of the initial P_i burst (Chock & Eisenberg, 1979), it would not be surprising if a fraction of the HMM was denatured in such a way that it still hydrolyzed ATP but did not show ^{18}O exchange. Although we do not understand why 20% of the HMM shows no exchange, we think that it would be unduly speculative to assume that these data require a new model for the mechanism of ATP hydrolysis. This is particularly the case because the data can also be explained if 20% of the HMM is denatured in some way. If this denaturation decreased the back-rate of the ATP hydrolysis step or increased the rate of P_i release at low actin

concentration, it would decrease the level of ^{18}O exchange shown by the denatured HMM. It should be noted that our data obtained with HMM are consistent with those of Shukla et al. (1988) although those authors offer interpretations using differing assumptions. Shukla et al. argue that the fraction of HMM in the high- and low-exchange classes varies as a function of actin concentration and the P_c of each class of exchanger also varies as a function of actin concentration.

Although under most conditions we can fit the ^{18}O exchange data to a single pathway, we can, of course, also fit these data to two (or more) pathways of exchange, and this raises an important point in interpreting ^{18}O exchange data. Some workers have found that, under a particular condition, it is necessary to fit the HMM data to two pathways of exchange and then, on this basis, have fit all of their data to two pathways of exchange without determining if this is, in fact, necessary under most conditions. In our view, an attempt should always be made, first, to fit the ^{18}O exchange data to a single pathway of exchange. Only if this fails should two pathways of exchange be considered, and even then it should be assumed that one of these pathways is identical with the ^{18}O exchange observed with S-1. Only after these conservative assumptions are made and it is clearly demonstrated that a significant fraction of the HMM is acting differently from S-1 should new models for the actomyosin ATPase activity be considered.

Although we do not think that HMM, in general, shows two pathways of ^{18}O exchange, it is clear that in skinned fibers or with actomyosin in vitro, two or more pathways of exchange are present. These data have been interpreted qualitatively in terms of the presence of force-producing bridges, but a full understanding of ^{18}O exchange in these complex systems would certainly be aided if we understood the mechanism of ^{18}O exchange in the simpler acto·S-1 system. The difficulty in these systems has been that there is still not agreement on the mechanism of the acto·S-1 ATPase activity, in particular, the nature of the rate-limiting step in the acto·S-1 ATPase cycle.

In this paper, we showed that the ^{18}O exchange data cannot be used to distinguish between the four-state and six-state kinetic models for the actomyosin ATPase activity. The data fit the four-state model if the rate-limiting step is the ATP hydrolysis step followed by a fast P_i release step. Since the P_i release step is fast compared to the back-rate of the ATP hydrolysis step, the model predicts that the level of ^{18}O exchange will decrease to very low values at high actin concentration, just as is observed experimentally.

Although the data fit the four-state model, they do not fit the six-state model if P_i rotation occurs at a rapid rate. In the six-state model, the rate-limiting step follows the ATP hydrolysis step, and this step is not fast enough, in comparison to the back-rate of the ATP hydrolysis step, to explain the low level of ^{18}O exchange observed at high actin concentration or with cross-linked acto·S-1. However, it is possible to fit the ^{18}O exchange data to the six-state model if we assume that the amount of ^{18}O exchange is limited by the rotation of P_i at the active site rather than the back-rate of the ATP hydrolysis step. In the presence of actin, the back-rate of the ATP hydrolysis is 27 s^{-1}. If we assume that the rate of P_i rotation is about 3 s^{-1} at saturating actin concentration, then we can explain the low level of ^{18}O exchange which occurs at high actin concentration even though the rate-limiting step follows the ATP hydrolysis step.

Limiting the rate of P_i rotation would also allow us to fit the ^{18}O exchange data to the four-state model with P_i release the rate-limiting step. However, this model cannot explain the large difference in K_{ATPase} and $K_{binding}$ which occurs at low ionic strength, and, therefore, if the four-state model is valid, it is most likely that the ATP hydrolysis step itself is rate limiting.

In conclusion, our study of ^{18}O exchange suggests that only one pathway of ^{18}O exchange probably occurs with S-1 and HMM in vitro, but our data cannot resolve whether the four-state or the six-state kinetic model of the actomyosin ATPase activity is valid. The data do show that for the six-state model to be valid, there must be a limit in the rate of P_i rotation at the active site to explain the low level of ^{18}O exchange observed at high actin concentration. Interestingly, if the four-state model is valid, the same rapid release of P_i which causes K_{ATPase} to be much larger than $K_{binding}$ also explains the low level of ^{18}O exchange observed. Recently, with porcine cardiac muscle, a large difference in K_{ATPase} and $K_{binding}$ has been observed, but much more ^{18}O exchange occurs in this system than in the skeletal system. The implications of these data for the four-state and six-state kinetic models will be discussed in the following paper (Stein et al., 1989).

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Oxygen Exchange Kinetics of Porcine Cardiac Acto–Subfragment 1†

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ABSTRACT: Recent studies have shown that the K_{ATPase} of porcine cardiac S-1 is severalfold stronger than $K_{binding}$. As with skeletal S-1, the four-state model can only explain this observation with the assumption that the release of the products of hydrolysis is rapid and not rate limiting. However, if the release of products is fast, the four-state model predicts that the extent of oxygen exchange with porcine cardiac S-1 should fall toward zero at high actin concentrations, as previously observed with skeletal acto–S-1. In the current work, we show that, in fact, the extent of oxygen exchange for porcine cardiac S-1 remains significant even at infinite actin concentration (i.e., with cross-linked actin–S-1) and that, therefore, the four-state model cannot adequately account for the oxygen exchange data and the ratio of $K_{binding}$ to K_{ATPase} simultaneously. As in the skeletal case, in order for the six-state model to account for these data, it is necessary to assume that P_i rotation in the acto–S-1·ADP·P_i state is rate limiting for oxygen exchange.

During the last 2 decades, there has been a great deal of interest in the biochemical kinetics of the skeletal myosin subfragment 1 (S-1)[1] ATPase activity (Taylor, 1979; Eisenberg & Greene, 1980; Sleep & Smith, 1981; Webb & Trentham, 1983). This work has led to the development of two possible models for the actin activation of the S-1 ATPase activity, a four-state model and a six-state model (Figure 1) (Stein et al., 1981). While there is a general consensus that a four-state model is the minimal kinetic model which can explain the actomyosin ATPase activity, there is still controversy over whether this model can actually account for all of the available steady-state and pre-steady-state data (Stein, 1988).

Data that are central to the argument against a four-state model are the difference observed between K_{ATPase}, the actin concentration where half-maximal acto–S-1 ATPase activity occurs, and $K_{binding}$, the actin concentration where half-maximal binding of the S-1–ATP complex to actin occurs. Stein et al. (1981) had noted a 4–8-fold difference between these constants for the skeletal myosin isoenzyme (A-1)S-1, and when the four-state and six-state models are required to account for this difference, they predict very different magnitudes of the phosphate burst at infinite actin concentration (Stein et al., 1984). The four-state model predicts that the magnitude of the P_i burst will fall toward zero as the actin concentration is increased, while the six-state model predicts that there will be a significant burst magnitude even at saturating actin concentration. Unfortunately, measurement of the magnitude of the phosphate burst at high actin concentrations is very difficult, and, therefore, there is still controversy over whether this magnitude is consistent with the four-state or the six-state model (Stein, 1988).

The reason that the four-state model predicts a very low value for the magnitude of the P_i burst at high actin concentrations is that this model can only account for the large difference between K_{ATPase} and $K_{binding}$ if the ATP hydrolysis step with actin bound is rate limiting and the P_i release step is very rapid. The four-state model therefore predicts that, during steady-state hydrolysis of ATP, state A·M·ADP·P_i will decompose much more rapidly than it is formed and will not be occupied to a significant extent. If state A·M·ADP·P_i is not occupied to a significant degree, the four-state model also leads to the prediction that almost no ^{18}O exchange should occur at high actin concentration. ^{18}O exchange requires that many reversals occur between state A·M·ADP·P_i and A·M·ATP before P_i is released into the solution. If the P_i release step is very rapid compared to the reverse rate of the ATP hydrolysis step, P_i release will occur before any reversals of the ATP hydrolysis step can take place, and, therefore, no ^{18}O

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[1] Abbreviations: S-1, myosin subfragment 1; A, actin; V_{max}, maximal ATPase rate per myosin residue; K_{ATPase}, actin concentration where half the maximal ATPase rate (V_{max}) is achieved; $K_{binding}$, actin concentration necessary to achieve half S-1 bound, during steady-state hydrolysis of ATP; M, myosin subfragment 1; P_i, inorganic phosphate; ATP, adenosine 5′-triphosphate.

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