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Comparative Studies of Oxygen Exchange Catalyzed by Myosin, Heavy Meromyosin, and Subfragment 1. Evidence That the γ-Phosphoryl Group of Adenosine Triphosphate Binds to Myosin in the Region of the (Subfragment 1)–(Subfragment 2) Hinge†

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ABSTRACT: At an intermediate stage in the hydrolysis of Mg^{2+}–ATP by myosin there is an extensive exchange of oxygen between water and the γ-phosphoryl group of the bound nucleotide. The exchange appears to result from a repeated cycle of cleavage and reverse cleavage of the bound ATP, with different oxygens coming into an exchanging position by rotation of the bound phosphate group. Actin activates the overall rate of hydrolysis at a rate-limiting step which follows the exchange reactions; thus, it decreases the time available for exchange when it decreases the turnover time of hydrolysis. By measuring exchange as a function of turnover time, which can be varied by changing the concentration of actin, it is possible to estimate the rate constants for oxygen exchange. Such estimates have indicated that the rate of rotation limits the rate of exchange. With myosin, not only is the average rate of exchange relatively slow, indicating considerable restriction in rotation, but only three of the four oxygens per P_i molecule are able to enter the exchange cycle. Apparently, one of the oxygens is bound to the protein. Comparative studies of oxygen exchange catalyzed by myosin and various single and double-headed fragments of myosin, reported here, suggest that the binding, which limits the extent and rate of oxygen exchange, depends on the integrity of the (S-1)–(S-2) hinge, the flexible region that connects each head of myosin to the body of the molecule. When this hinge is cleaved in the preparation of subfragment 1, then even the fourth oxygen can exchange at a measurable rate, and the average rate constant for exchange of the other three is significantly increased. The results have led us to postulate that one oxygen in the γ-phosphoryl group of ATP, the one that cannot exchange, is bound to myosin in the region of the (S-1)–(S-2) hinge, and that this binding serves to connect points on the flexible hinge to points of the active site that catalyze hydrolysis. An hypothesis is presented that describes how, in this way, changes at the active site, which occur in the course of hydrolysis, could cause the broader movements of the myosin head that occur during contraction.

It has been known for some time that oxygen atoms initially on the γ-phosphoryl group of ATP exchange with oxygens of water at some intermediate stage of Mg^{2+}–ATP hydrolysis by myosin (Levy and Koshland, 1958, 1959; Levy et al., 1960, 1962; Sartorelli et al., 1966). It now appears that this reaction, called intermediate exchange, occurs at step 3 in the following enzymatic pathway (Bagshaw and Trentham, 1974; Bagshaw et al., 1974; Lymn and Taylor, 1971).

$$\mathrm{M} + \mathrm{ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{M{\cdot}ATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} \mathrm{M^{**}{\cdot}ADP{\cdot}P_i}$$

$$\underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ADP{\cdot}P_i} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftharpoons}} \mathrm{M^{*}{\cdot}ADP{\cdot}}$$

$$+ \mathrm{P_i} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} \mathrm{M{\cdot}ADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} \mathrm{M} + \mathrm{ADP}$$

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In this pathway, M, M*, and M** represent different conformations of myosin distinguished by levels of fluorescence. Step 3, k_{+3}, involving intermediates that show extensive oxygen exchange (Bagshaw et al., 1975) is called the cleavage step, and M**·ADP·P_i[1] is written to indicate tightly bound products of the reaction. Since the reverse cleavage step, k_{-3} ($\geq 18\ s^{-1}$) is relatively fast compared with the rate-limiting step, k_{+4} ($0.06\ s^{-1}$), a cycle of cleavage and reverse cleavage occurs many times before a P_i molecule is released. As proposed by Bagshaw and Trentham (1973), such cycling could account for intermediate exchange if the oxygen atom lost from the P_i through reverse cleavage could be different from the one added to the γ-phosphoryl group during cleavage. For this to happen requires only that the oxygens around the phosphorus atom have some chance to change their relative positions at the active site, e.g., by the actual rotation of the bound P_i in M**·ADP·P_i.

An alternate mechanism for intermediate exchange has been proposed by Young et al. (1974). In their mechanism, step 3 is not actually a cleavage reaction. Instead, they postulate that both M*·ATP and M**·ADP·P_i are pentacoordinate intermediates (with five oxygens around the terminal phosphorus atom), formed by the addition of water to the bound ATP. The water is added at step 2 to form M*·ATP and this then transforms into M**·ADP·P_i by the bending and spatial transposition of the five oxygen–phosphorus bonds around the terminal phosphorus atom. As a result of this process, called pseudorotation, the bond connecting ADP to the γ-phosphoryl group is weakened and this favors the splitting of M**·ADP·P_i at a subsequent step (which would have to be added to the above pathway). In this pseudorotation mechanism, Young et al. suggest that two oxygens in the form of oxoniums may exchange with water at the M*·ATP stage. But to account for the observed exchange of three to four oxygens per P_i molecule, their mechanism, in common with that of Bagshaw and Trentham, also requires, effectively, some rotation of oxygens at the active site. Thus, whatever the exact mechanism of cleavage, there is good evidence that exchange is closely related to step 3 and its reversal and depends to some extent on a spatial rearrangement of oxygens at the active site.

In our previous paper (Shukla and Levy, 1977a) we described a method for estimating the apparent rate constants for oxygen exchange. To do this, we took advantage of the fact that actin, depending on its concentration, can increase the turnover rate for ATP hydrolysis up to 100-fold or more without directly affecting what we call the cleavage cycle (i.e., step 3 and its reversal). It appears from the kinetic studies of Lymn and Taylor (1970, 1971) that actin does this by forming an actin–M**·ADP·P_i complex which rapidly goes through step 4 at a rate of 10–20 s^{-1}. Thus, actin effectively removes M**·ADP·P_i from the cleavage cycle and its associated intermediate exchange. As the concentration of actin is increased, the average turnover time for hydrolysis is shortened and, on average, less time is available for the cleavage cycle and oxygen exchange. An estimate for $k_{exchange}$ can thus be made by relating the extent of ^{18}O incorporation to the turnover time as set by the actin concentration.

The results of such an experiment with subfragment 1 (S-1) (Shukla and Levy, 1977a) indicated that after one labeled oxygen (from $H_2{}^{18}O$) was incorporated into the P_i molecule by the fast cleavage reaction ($k_{+3} \geqq 160\ s^{-1}$), the other three oxygens exchanged with an average apparent rate constant of $1.2\ s^{-1}$. However, after two of these oxygens were labeled, the exchange proceeded with an average apparent rate of only 0.2 s^{-1}. Even the faster initial rate was much slower than that expected if k_{-3} were the rate-limiting step in the exchange mechanism. Therefore, we suggested that the rate of rotation, k_{rot} (not k_{-3}), limited exchange, and that binding of one oxygen to the protein severely restricted its rotation. Using this same approach we have continued these studies on myosin itself and on various enzymatically active fragments of myosin. A comparison of the rate and extent of oxygen exchange catalyzed by single-headed (subfragment 1) and double-headed (myosin, heavy meromyosin) forms has supported the earlier indication that there is an intrinsic difference in their exchange properties (Shukla and Levy, 1977b). In myosin, and in certain double-headed fragments, one oxygen per P_i molecule is unable to exchange at a measurable rate; the other three, although apparently restricted in their rotation, are able to exchange. By comparison, in single-headed fragments, even the fourth oxygen is able to exchange at a measurable rate, and the other three, apparently with more freedom to rotate, exchange at a faster rate than in myosin. If, as we have suggested, one oxygen in each P_i molecule is bound to the protein, then these results indicate that this binding depends on the integrity of what we call the (subfragment 1)–(subfragment 2) hinge (the apparently flexible segment of the myosin molecule that connects the two heads to the body). When this hinge is cleaved by proteolytic enzymes, in the preparation of S-1, the binding of the phosphate oxygen is weakened. Thus, there is greater rotation of bound P_i, and faster intermediate oxygen exchange.

The findings have led us to postulate that the oxygen binding site, which is made apparent by these oxygen exchange studies, serves to directly connect the γ-phosphoryl group of ATP to the moveable hinge of the myosin molecule. In our hypothesis, this connection directly couples enzymatic changes at the active site to conformational changes at the flexible hinge. Thus, steps in the cleavage of ATP at the active site can be linked, through the substrate, to relatively small conformational changes at the hinge. These changes, e.g., a change in angle at the hinge, can then be translated into the broader movements of the myosin head that are believed to pull the actin filament during contraction.

Experimental Procedures

Protein Preparations. Rabbit back and leg muscle was used for all protein preparations. Actin was extracted from acetone-dried muscle powder (Szent-Gyorgyi, 1951) by the method of Spudich and Watt (1971). Myosin was prepared essentially as described by Mommaerts and Parrish (1951). However, a step involving ammonium sulfate fractionation was added to obtain actin-free myosin; the purified myosin that precipitated between 40 and 50% saturation was used for the kinetic studies involving myosin itself and also to prepare the myosin fragments. Myosin depleted of the DTNB light chain (containing about 50% of the normal amount) was prepared by the method of Bagshaw (1977).

Three types of subfragment 1 (S-1) were prepared. One (Mg^{2+}–S-1) containing all three light chains of myosin was prepared by the method of Margossian et al. (1975). The others, each containing only one alkali light chain, A1 or A2, were prepared essentially as described by Weeds and Taylor (1975), except that, on our column of Whatman DE 32, S-1 A1 (containing only the A1 chain) was eluted by the buffer itself before the salt gradient was started; then S-1 A2 (containing only the A2 chain) was eluted with the salt gradient.

Heavy meromyosin (HMM) was prepared by digesting myosin with trypsin or chymotrypsin. Tryptic HMM (HMM-T) was prepared by the method of Holt and Lowey

[1] Abbreviation used: P_i, inorganic phosphate.

(1975). Chymotryptic HMM was prepared by the method of Weeds and Taylor (1975), digestion being carried out in the presence of high salt (HMM-C), and by the method of Bagshaw (1977), when the digestion was carried out in low salt in the presence of 5 mM Ca^{2+} (Ca^{2+}-HMM-C). Gel electrophoresis was carried out on 10% polyacrylamide gels with myosin and its subfragments (Weber and Osborn, 1969). The gels were scanned, using an attachment to the Beckman Acta II spectrophotometer, and the relative amounts of each light chain estimated from the records of optical density. The three light chains, A1, A2, and DTNB, appeared to be intact in myosin, HMM-T, Ca^{2+}-HMM-C, and Mg^{2+}-S-1, although DTNB light chain seemed to travel faster and coincided with A2 in HMM-T and Ca^{2+}-HMM-C. There appeared to be a substantial loss of DTNB light chain in HMM-C. The subfragments, S-1 A1 and S-1 A2, did not contain any DTNB light chain. For the calculations of turnover time, the following equivalent weights per head were used: S-1 A1, 105 000; S-1 A2, 100 000; Mg^{2+}-S-1, 120 000; HMM-T and Ca^{2+}-HMM-C, 170 000; HMM-C, 150 000; and myosin, 230 000.

Measurement of Adenosine Triphosphatase Activity. A typical $H_2{}^{18}O$ reaction mixture contained 25 mM KCl, 25 mM Tris, pH 7.4, 5 mM $MgCl_2$ and was made in the following manner. Solid Tris, KCl, and $MgCl_2$ were dissolved in $H_2{}^{18}O$ (approximately two-thirds of the final volume) and the pH was adjusted to 7.4 with concentrated HCl. Appropriate concentrations of actin and the enzyme, S-1 or HMM, were then added. The resulting solution was thoroughly mixed in a hand homogenizer. Thorough mixing at this stage was particularly important where high concentrations of actin formed a viscous jelly. The temperature of the reaction mixture was brought to 23 °C and the reaction was started by adding ATP to a final concentration of 5–6 mM. After adding ATP, the reaction solution was again mixed thoroughly in a hand homogenizer.

The concentration of actin varied between 0 and 20 mg/mL. In each experiment, the concentration of myosin, HMM or S-1, between 0.08 and 2.3 mg/mL was adjusted to the specific rate of hydrolysis (set by the actin concentration) so that 80–100% of the ATP was hydrolyzed in less than 1 h, usually in less than 15 min. Some time after the start of the reaction, 1 mL of the reaction mixture was withdrawn and frozen. This was later used for determining the ^{18}O content of the medium. Periodically, during the course of the reaction, 0.5 mL aliquots of the reaction mixture were removed and assayed for inorganic phosphate (Koshland and Clarke, 1953). These assays were used to generate the rate curves for ATP hydrolysis, from which turnover times were calculated. After the reaction in $H_2{}^{18}O$ had proceeded to 80–100% hydrolysis, the reaction mixture was chilled in an ice bath and quenched with sufficient trichloroacetic acid to make a final concentration of 10%. The acidified reaction mixture was treated with acid-washed charcoal (10 mg per μmol of nucleotide) for 10 min at 0 °C to remove nucleotides. The denatured protein and charcoal were then removed by vacuum filtration through a small Buchner funnel. The resulting filtrate was again treated with the charcoal for 30 min at 0 °C and filtered again. The P_i content of the filtrate was determined. Solid carrier KH_2PO_4 was added to bring the total amount of P_i to about 0.7 mmol. The P_i content was again determined to obtain an appropriate dilution factor.

The P_i was precipitated as $MgNH_4PO_4$, dried with alcohol and ether, and ultimately isolated as KH_2PO_4 essentially as described by Dempsey et al. (1963). KH_2PO_4 or medium water was converted to CO_2 by heating with 100 mg of guanidine hydrochloride for about 8 h at 250 °C in a sealed vial according to the method of Boyer and Bryan (1967). This CO_2 sample was then collected and analyzed for its isotopic ratio [46/(44 + 45)] on the mass spectrometer. The steps of our current procedure are described in detail elsewhere (Shukla, 1977).

Despite the low ionic strength, it proved possible to study myosin at different levels of actin, using the same approach we originally developed with soluble fragments. This was so because the mixtures of actin and myosin remained in solution at the high concentrations of ATP used in these studies. Furthermore, to assure that the proteins did not form any insoluble complex or undergo any superprecipitation, the reactions with intact myosin were started by adding the actin a few seconds after the addition of ATP rather than by adding ATP to a mixture of the proteins, as in the usual procedure.

Results

As described in the introduction, the rate of incorporation of ^{18}O from water into enzyme-bound P_i, during ATP hydrolysis, can be estimated by determining the ^{18}O content of the P_i as a function of turnover time (set by the actin concentration). However, such an estimate of the ^{18}O incorporation rate does not give the apparent rate constant for exchange, k_e, since it does not take into account the exchange reactions that occur between ^{18}O in the water and ^{18}O already incorporated into P_i by prior reactions. To correct for this recycling of the label, it is necessary to formulate a rate equation from which k_e can be calculated. The approach we have taken is essentially the same as that described by Levy and Koshland (1959). It is considered that, during exchange, the initial ^{16}O in the P_i is lost exponentially; that is, that the fractional rate of loss of ^{16}O (in exchange for ^{18}O) at any moment is a function of the fractional content of ^{16}O in the P_i at that time. This gives the equation:

$$\frac{-d(1-X)}{dt} = k_e(1-X) \qquad (1)$$

where X is the fraction of labeled ^{18}O in the P_i and, therefore:

$$\ln(1-X) = -k_e t + C \qquad (2)$$

where C is a constant, and where t is the lifetime of the exchanging intermediate. Taking the turnover time as t, values of k_e can be calculated (Table I) or obtained graphically, as in Figure 1, by plotting $\ln(1-X)$ against t. The slope of such a plot is $-k_e$. In such a plot, the value for $(1-X)$ at the ordinate is 0.75 instead of 1, because at virtually zero time, one ^{18}O is added to the P_i by the first cleavage reaction ($k_{+3} \geqq 160\ s^{-1}$). In the simplest case, if all oxygens exchanged with the same rate constant, the plot would be a straight line intercepting the ordinate at 0.75.

The curves in Figure 1 indicate that, in fact, not all the oxygens in each p_i molecule exchanged at the same rate. As shown in the upper curve, when the reaction was catalyzed by myosin itself, or by certain double-headed fragments, one oxygen in each P_i molecule apparently did not exchange. The inability of this oxygen to take part in the exchange cycle is reflected in the flat portion of the curve after three oxygens have been labeled; thus, between 3 and 25 s of turnover time, the fraction of unlabeled oxygen remaining in the P_i was, within experimental error, 0.25, or one oxygen per P_i. Since the rate constant for this one oxygen is practically zero, the average rate constant for the other three can be estimated from the half-time for going from three unlabeled oxygens at zero time to the one that cannot exchange, or, by calculation using eq 3 to be described. Either way, the value for k_e of the three

TABLE I: Estimated Rate Constants for Oxygen Exchange by Myosin and Various Fragments of Myosin.

Protein (symbol)	Light chains present A1	A2	DTNB	Conditions[d] Enzyme	Salt concn	Divalent cation concn	Turnover time (t, s)	Fraction of unlabeled O remaining in P_i ($1 - X$)	k_e (s^{-1})[e] calcd for 3 exch. O per P_i molecule[a]	4 exch. O per P_i molecule[b]
Myosin	+	+	+				0.48	0.53	1.2	0.7
							0.48	0.54	1.1	0.7
Heavy meromyosin (HMM-T)	+	+	+	Trypsin	0.5 M KCl	None	0.27	0.62	1.1	0.7
							0.53	0.55	1.0	0.6
							0.67	0.47	1.2	0.7
							1.30	0.37	1.1	0.5
Heavy meromyosin (HMM-T)	+	+	+	Trypsin	0.5 M KCl	None	0.34	0.56	1.4	0.9
							0.51	0.44	1.9	1.0
							0.67	0.42	1.6	0.9
							1.20	0.39	1.1	0.5
							1.45	0.31	1.5	0.6
Heavy meromyosin (HMM-T)	+	+	+	Trypsin	0.5 M KCl	None	0.42	0.46	2.1	1.2
							0.44	0.45	2.1	1.2
Heavy meromysin (Ca^{2+}-HMM-C)	+	+	+	Chymo-trypsin	0.12 M NaCl	5 mM Ca^{2+}	0.54	0.45	1.7	0.9
Heavy meromyosin (HMM-C)	+	+	c	Chymo-trypsin	0.6 M NaCl	None	0.48	0.41	2.4	1.2
							0.46	0.40	2.5	1.3
							0.45	0.41	2.4	1.3
Subfragment 1 (Mg^{2+}-S-1)	+	+	+	Papain	0.12 M KCl	2 mM Mg^{2+}	0.28	0.49	2.6	1.5
							0.40	0.44	2.4	1.3
							0.72	0.32	2.7	1.2
							0.72	0.33	2.5	1.1
							0.94	0.29	2.7	1.0
Subfragment 1 (S-1 A1)	+	−	−	Chymo-trypsin	0.12 M NaCl	None	0.34	0.44	2.8	1.6
							0.54	0.32	4.2	1.6
							0.60	0.32	3.2	1.4
Subfragment 1 (S-1 A2)	−	+	−	Chymo-trypsin	0.12 M NaCl	None	0.41	0.36	3.7	1.8
							0.49	0.32	4.0	1.7
							0.50	0.33	3.6	1.7

[a] See eq 3 in the text. [b] See eq 4 in the text. [c] Contained less than half the normal amount of DTNB light chain. [d] Method of preparation conditions for proteolytic digestion of myosin. [e] Estimated rate constant of oxygen exchange.

exchangeable oxygens is about 1.2 s^{-1}. The results indicate that, in myosin and certain heavy meromyosins, one oxygen in each P_i molecule is prevented from entering the exchange cycle; that is, it cannot take the position at the active site from which oxygen leaves the P_i during reverse cleavage (k_{-3}). The simplest interpretation, which we presented in the earlier paper of this study (Shukla and Levy, 1977a) is that this one oxygen is bound to the protein; in myosin, the other three oxygens in the same P_i, although restricted, are able to rotate at a relatively slow rate which allows their complete exchange in about 3 s.

For comparison with myosin and the double-headed fragments that exchange three oxygens per P_i molecule, the lower curve in Figure 1 shows that all single-headed fragments (and one preparation of HMM) catalyzed the exchange of four oxygens per P_i molecule. This difference is also shown by the data of Table II. The apparent average rate constant for exchange of these four oxygens can be estimated graphically from the apparent half-time for going from three unlabeled oxygens at zero time (0.75 on the ordinate) to less than 0.5 unlabeled oxygen (<0.1 on the ordinate) after about 3 s (bottom curve, Figure 1); or the constant can be calculated, using eq 4 to be described. Either of these estimates, however, which treats all four exchangeable oxygens together, gives a lower limit for the average rate of exchange by three of them. This follows from indications that, even in these single-headed fragments, one oxygen per P_i molecule (probably corresponding to the tightly bound oxygen in intact myosin) exchanges at a relatively slow rate compared with the other three. This is evident for Mg^{2+}-S-1 by the shallow slope of the bottom curve at long turnover times. Within the error of the measurements, the points forming this section of the curve are significantly above zero. Nevertheless, as is evident, estimating the rate of the slow exchange from these points has in it a high degree of uncertainty. Moreover, the extensive data needed for this were obtained only for Mg^{2+}-S-1. Therefore, to compare all the fragments with each other, we have used an alternate approach. In this, the average apparent rate constant of exchange was calculated for two boundary conditions: (1) when only three of the four oxygens are able to exchange; and (2) when all four oxygens exchange at an equal rate. For the first condition where the fraction of ^{16}O in P_i is 0.75 at $t = 0$ and 0.25 at $t > 3$ s, eq 2 becomes:

$$\ln \frac{0.5}{(0.75 - X)} = k_e{}^3 t \qquad (3)$$

where $k_e{}^3$ is the average rate constant for the three exchangeable oxygens per P_i molecule. For the second condition, where, again, the fraction of ^{16}O in P_i is 0.75 at $t = 0$, but is zero at $t > 3$ s, eq 2 becomes:

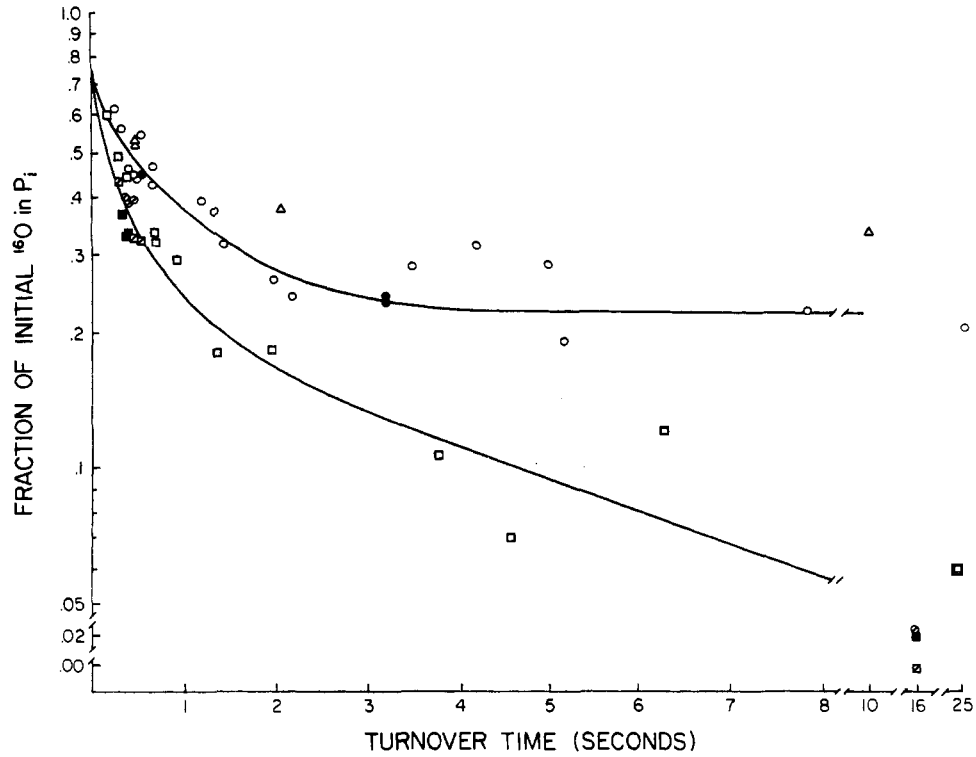

FIGURE 1: Rates of oxygen exchange by myosin and various fragments of myosin as indicated by the loss of the initial unlabeled oxygen in P_i as a function of turnover time. Conditions: 25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris buffer, pH 7.4, 5-6 mM ATP in $H_2{}^{18}O$, 23 °C. Protein concentrations were adjusted as explained in Experimental Procedures. (Top) (open circles) HMM-T; (closed circles) Ca^{2+}-HMM-C; (open triangles) myosin. (Bottom) (open squares) Mg^{2+}-S-1; (cross hatched squares) S-1 A1; (solid squares) S-1 A2. (Cross hatched circles) HMM-C. For a description of the various proteins, see Table I and Experimental Procedures.

TABLE II: The Extent of Oxygen Exchange Catalyzed by Myosin and Various Fragments of Myosin during Relatively Long Turnover Times.

Protein[a]	Actin concn (mg/mL)	Turnover time (t, s)	No. of labeled oxygens from water incorp per P_i molecule
Myosin	None	25	2.9 (2.56-3.27)[b]
Myosin	0.07	10	2.7
Myosin	0.27	2	2.7
Myosin	0.35	2	2.4
Myosin with ITP as substrate instead of ATP	None	2-3	2.9 (2.58-3.32)[c]
Myosin treated to remove the DTNB light chain	0.27	6	2.5
Myosin treated to remove the DTNB light chain	1.06	6	2.5
HMM-T	None	25	3.2
HMM-T	0.1-1.0	3-8	2.8-3.1[d]
HMM-T	0.35	5	3.2
Ca^{2+}-HMM-C	0.5	3	3.0
HMM-C	None	16	3.9
Mg^{2+}-S-1	None	25	3.8
S-1 A1	None	16	4.4
S-1 A2	None	16	3.9

[a] For description of protein and method of preparation, see Table I and Experimental Procedures. [b] Previously unpublished data; six different preparations, 17 different measurements. [c] Previously unpublished data; three different preparations, 13 different measurements. [d] Four different values, shown in Figure 1, bottom curve.

$$\ln \frac{0.75}{(1-X)} = k_e{}^4 t \qquad (4)$$

where $k_e{}^4$ is the average rate constant for four exchangeable oxygens per P_i molecule. The estimates of k_e calculated from these equations are given in Table I. For myosin and the double-headed fragments, the first condition is the more realistic; therefore, the values calculated for three exchangeable oxygens (the higher values in Table I) represent the best estimate for the actual average rate of exchange by three oxygens in the P_i molecule. For other fragments, in which the fourth oxygen exchanges at a measurable rate, the average rate constant for the three faster exchanging oxygens lies somewhere between the two estimates.

In the case of Mg^{2+}-S-1, as we have discussed, where sufficient data was available, k_e', the rate constant for the fourth oxygen, could be approximated. It appears to be about 0.2 s^{-1}, from the slope of the line drawn through the points between 2 and 6 s and extrapolated back to 0.25 at zero time. The average rate constant for the first three oxygens, then, can be

TABLE III: Comparative Values for the Rate and Extent of Oxygen Exchange Catalyzed by Myosin and Various Fragments of Myosin.

Protein[a]	Rate of oxygen exchange, No. of reactions per s per P_i molecule calcd for: 3 exchangeable oxygens[c] per P_i molecule	Rate of oxygen exchange, No. of reactions per s per P_i molecule calcd for: 4 exchangeable oxygens[d] per P_i molecule	Extent of oxygen exchange, No. of labeled oxygens from water incorp per P_i molecule (during long turnover times)[b]
Myosin	3.5	2.8	2.9
HMM-T	3.3	2.5	3.2
HMM-T	4.5	3.1	3.1
HMM-T	6.3	4.8	3.2
Ca^{2+}-HMM-C	5.1	3.6	3.0
HMM-C	7.3	5.1	3.9
Mg^{2+}-S-1	7.7	4.9	3.8
S-1 A1	10.2	6.1	4.4
S-1 A2	11.3	6.9	3.9

[a] For descriptions of proteins and methods of preparation, see Table I and Experimental Procedures. [b] For conditions of these measurements, see Table II. [c] See eq 6 in the text. [d] See eq 7 in the text.

estimated graphically by subtracting, from each point on the initial portion of the actual curve, the corresponding value on the extrapolated line. A plot of the logarithm of these differences against time gives a line with a slope equal to k_e for the three faster exchanging oxygens. The estimate of this value for Mg^{2+}-S-1 is about 2 s^{-1}. The equation that describes the line having a slope, k_e, equal to this value is:

$$\ln \frac{0.5}{(1 - X) - 0.25\, e^{-k_e' t}} = k_e t \qquad (5)$$

where k_e', in this case equal to 0.2 s^{-1}, is the rate constant for the slow exchange of one oxygen per P_i molecule. Equation 5 gives eq 3 when $k_e' = 0$, and it gives eq 4 when $k_e' = k_e$. For Mg^{2+}-S-1, the rate constant, k_e, from eq 5 is closer to the higher estimate in Table I which assumes only three exchangeable oxygens; this is to be expected, since the slow rate of exchange by the fourth oxygen would make relatively little contribution to the average exchange. However, for some of the other fragments, as the measured rate of exchange gets faster, it is possible that the rate of exchange by the fourth oxygen becomes comparable to that of the other three. If so, the real value for the rate of exchange by all the oxygens would be closer to the lower estimate. The rate of exchange can be expressed more mechanistically in terms of each bound P_i molecule, by multiplying the estimated rate constant for exchange times the number of exchangeable oxygens in each P_i. Thus for three exchangeable oxygens:

$$N = k_e^3 \times 3 \qquad (6)$$

where N is the number of exchange reactions in s^{-1} P_i^{-1}. For four exchangeable oxygens, the value for N is given by:

$$N = k_e^4 \times 4 \qquad (7)$$

The value of N gives an estimate of the rate of rotation if, as we postulate, the rotation of the bound P_i limits the rate of exchange. Calculated values for N of myosin and various fragments are given in Table III.

It is significant that with the fastest exchanging fragments, S-1 A1 and S-1 A2, even the lowest estimate of exchange from eq 7 (assuming all four oxygens are free to exchange at the same rate) is twice the rate calculated for the three exchangeable oxygens of myosin from eq 6 (Table III). This indicates that the difference in the apparent exchange is not just the result of an increase in the number of exchanging oxygens from three to four. Instead, the results support the idea that, when the fourth oxygen becomes less restricted and can exchange at a measurable rate in the single-headed fragments, then the rate of rotation and, therefore, exchange of the other oxygens in the P_i molecule also increases significantly. In fact, the rate of exchange with certain of the fragments (e.g., S-1 A1 or S-1 A2 in Table III) is sufficiently high to indicate that in these instances the rate-limiting step might be k_{-3} itself rather than rotation.

Comparing all the different preparations, the slowest oxygen exchange, the greatest similarity to myosin, was observed in the double-headed fragments prepared with trypsin, an enzyme which selectively cleaves HMM from myosin and appears to do least damage to the (S-1)-(S-2) hinge and the DTNB light chain. The highest rates of exchange were observed in the single-headed fragments prepared with chymotrypsin, an enzyme which attacks the (S-1)-(S-2) hinge and the DTNB light chain in the absence of divalent cation. In the fragments (S-1 A1 or S-1 A2) that were made this way and which showed very high rates of exchange, the DTNB chain was absent. Parenthetically, it made no significant difference which of the alkali light chains the fragment contained. It appears now that these different chains represent different isoenzymes of myosin (Holt et al., 1977).

Between those heavy meromyosin fragments with exchange properties resembling myosin and those subfragment 1 preparations that exchange most rapidly, there were forms that showed intermediate rates and levels of oxygen exchange (summarized in Table II). Although, as we have emphasized, the double-headed fragments, in general, resembled myosin, the HMM made with chymotrypsin in high salt, but with no divalent metal, (HMM-C) showed a rate and extent of exchange more similar to the single-headed forms than to myosin itself. Apparently, each head of this preparation acted, with regard to oxygen exchange, as if it were free, even though it remained physically connected to its partner; this preparation of HMM had less than half the normal content of DTNB light chain. Yet, when about half of the DTNB light chain was removed from myosin, this had no significant effect on oxygen exchange (Table II).

In general, the single-headed fragments exchanged at a faster rate. However, there was a significant difference in rate between those prepared with chymotrypsin in the absence of divalent metal ion, S-1 A1 or S-1 A2 (containing no DTNB), and those prepared with papain in the presence of Mg^{2+}, which protected the DTNB chain. The latter, Mg^{2+}-S-1, showed the slowest exchange by a single-headed form, suggesting that the site that restricts oxygen exchange, although substantially modified in this preparation, retained some of the original capacity to limit exchange. Taken together the comparative

studies indicate that the inhibition of oxygen exchange by myosin depends on the integrity of the (S-1)-(S-2) hinge and that cleavage or damage to the hinge is often associated with damage to the DTNB light chain.

Discussion

Studies on the kinetics of oxygen exchange reported here and in the previous papers lead us to the following main conclusions.

(a) During the hydrolysis of a molecule of Mg^{2+}-ATP at the active site of native myosin, only three of the four oxygens in the bound phosphate group of M**·ADP·P_i can exchange with the oxygen of water during the normal turnover time. The fourth oxygen cannot exchange significantly during this time because, we postulate, it is tightly bound to the protein at what we call the oxygen-binding site. Binding to this site prevents the fourth oxygen from entering the exchange cycle; it also contributes to a general restriction in the rotation of the bound P_i molecule and, therefore, decreases the rate of exchange of the other three oxygens.

(b) Certain preparations of HMM retain the full capacity of myosin to inhibit oxygen exchange. Apparently, the postulated oxygen binding site is conserved in these double-headed fragments. On the other hand, it appears to be lost or modified in single-headed fragments, which allow exchange of all four oxygens per P_i during the normal turnover time and catalyze a higher average rate of exchange. Thus, it seems, the oxygen binding site requires the integrity of the (S-1)-(S-2) hinge. When the hinge is cleaved in the preparation of S-1 there is a marked increase in the rate and extent of exchange.

(c) If it is assumed that each head hydrolyzes ATP at about the same rate, as indicated, e.g., by the same activity per head in myosin, heavy meromyosin, and subfragment 1 (Lowey, 1971; Moos, 1972), then it follows that both heads catalyze substantial exchange; this is necessary to account for the average measured exchange of three oxygen per P_i (3 O/P_i). The simplest assumption at this time is that each head catalyzes the exchange of 3 O/P_i. It is possible that one exchanges 2 O/P_i and the other 4 O/P_i but this is more complicated and there is no evidence for it at this time. Clearly, single-headed fragments of all types catalyze the exchange of 4 O/P_i. These indications that each head of myosin catalyzes oxygen exchange are consistent with the recent measurements of the phosphate burst by Taylor (1977); his data indicate that each head forms the M**·ADP·P_i intermediate during hydrolysis, and, as we have discussed, it is this intermediate that appears to play a direct role in the exchange cycle.

(d) Although the maximum extent of oxygen exchange catalyzed by myosin indicates that both heads take part in these reactions, the relatively slow rate of exchange by myosin and certain double-headed fragments, which is about half the rate observed with single-headed forms, may be a clue to some cooperative inhibition of exchange by the two heads. As we suggested in an earlier note (Shukla and Levy, 1977b), the fact that myosin or HMM exchanges at about half the rate per head as does S-1 is consistent with the possibility that exchange, although it occurs on both heads, does so on only one head at a time; this would effectively reduce by half the time available for exchange and result in a 50% reduction in the apparent rate constant. In this kind of mechanism (which might be related to a mechanism that coordinates the operation of the two heads in contraction), both the relatively fast exchange of three oxygens per P_i molecule and the slow exchange of the fourth protein-bound oxygen would be reduced by half in myosin compared with S-1. Accordingly, given sufficient time, it would be expected that even myosin must exchange all four oxygens. In fact, there are values in the literature for the extent of oxygen exchange by myosin which are significantly greater than three O/P_i. And recently we have observed values close to four with purified myosin preparations having a long turnover time (40–50 s). In general, it appears, within the accuracy of the measurements, that only three of four oxygens exchange during a turnover time of up to 25 s or so (see Table II), but that, when the state of the protein (or other factors not yet defined) increases the turnover time, then the fourth oxygen is also exchanged.

(e) There is evidence that the DTNB light chain is in the proximity of the (S-1)-(S-2) hinge, as suggested by Werber et al. (1972) who noted that cleavage of the hinge and the loss or cleavage of the DTNB light chain appear to go together. Recently, Bagshaw (1977) has shown that the binding of divalent metal ion to the DTNB light chain protects this hinge from cleavage by chymotrypsin; earlier Margossian et al. (1975) had shown that magnesium protected the DTNB light chain itself during the preparation of S-1 with papain. It therefore seems of some potential importance that the one preparation of HMM that catalyzed oxygen exchange similar to the single-headed preparations contained substantially less than normal amounts of the DTNB chain, and that the single-headed fragments that exchanged most rapidly were those without this chain. This appears to reflect the fact that the DTNB chain and the (S-1)-(S-2) hinge are close together so that the damage to the hinge, which affects oxygen exchange, is correlated with damage to the chain. At one point in the course of this study, it seemed an intriguing possibility that the DTNB light chain with its bound metal ion was actually part of the oxygen-binding site we have postulated. To test this, as mentioned in the results section, we compared oxygen exchange by native myosin and by myosin from which the DTNB chain was purposely removed; the results (see Table II) show that removal of about 50% of this chain without cleavage of the hinge had no significant effect on oxygen exchange.

Putting these ideas together leads us to the following summary and hypothesis. (1) One oxygen atom in the γ-phosphoryl group of ATP is tightly bound to an oxygen-binding site of myosin and remains bound in the M**·ADP·P_i intermediate; thus only 3 O/P_i can readily exchange. (2) The oxygen-binding site is in the region of the (S-1)-(S-2) hinge; thus fragments of myosin, in which the integrity of the hinge region has been compromised, bind this oxygen less tightly. This gives the bound P_i greater freedom to rotate and so allows a greater rate of oxygen exchange. (3) Each molecule of ATP is attached to myosin at two functionally different places: (a) at points of the enzymatic active site where cleavage of the terminal bond is catalyzed; and (b) at points of the oxygen-binding site in the region of the flexible (S-1)-(S-2) hinge. (4) The dual attachment by ATP connects the two main elements of the coupling system; thus, through the substrate, chemical events at the enzymatic site during the course of hydrolysis are linked to conformational changes in a flexible and strategic segment of the protein. With the ATP actually connected through its terminal oxygen to the oxygen-binding site at the hinge, it becomes easier to envision the chemical-physical coupling of the system. For example, the cleavage of ATP under such circumstances could directly move the hinge, or alter its angle, and this movement could be amplified at the crown of the myosin head where actin might attach. Such an operation of ATP at the hinge would be analogous to moving the short end of a lever.

It seems quite logical that the enzymatic and energetic changes would occur at or near the segment of the myosin molecule that appears most flexible, where conformational

changes are most likely to occur, and where those changes could have broad effects on the position of the head. This may, as we have proposed, be part of some cooperative interaction between the heads which is reflected in the relatively slow rate of oxygen exchange by double-headed forms (Shukla and Levy, 1977b). Also, the involvement of both ATP and the light chains at the hinge makes it easier to understand, e.g., how the action of calcium at one light chain on one head of certain invertebrate myosins affects the interaction of both heads with actin (Szent-Gyorgyi et al., 1973).

The hypothesis presented here predicts that any major disruption of the (S-1)–(S-2) hinge of myosin will increase oxygen exchange and interfere with contraction even though it allows uncoupled hydrolysis of ATP. This happens, it is postulated, because the attachment of the terminal phosphate group of ATP to the hinge plays an essential role in contraction, but is not necessary for hydrolytic cleavage. Thus, in myosin and certain preparations of HMM with the oxygen binding-site intact, the cleavage and rearrangement of ATP during steps in hydrolysis are directly and tightly linked to changes at the hinge that initiate movement of the heads. In single-headed forms, the ATP is hydrolyzed but the normal coupling to the hinge (now cleaved) is weakened or lost.

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