Mechanism of Oxygen Exchange in Actin-Activated Hydrolysis of Adenosine Triphosphate by Myosin Subfragment 1[†]

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ABSTRACT: The γ-phosphoryl groups of two intermediates (M*·ATP and M**·ADP·P_i) in the pathway of MgATP hydrolysis by myosin undergo extensive oxygen exchange with water. Actin activates the overall rate of hydrolysis at a rate-limiting step which follows these exchange reactions. Thus, actin, by decreasing the turnover time of hydrolysis, would be expected to proportionately decrease the time available for oxygen exchange. Using subfragment 1 of myosin, the turnover time of hydrolysis can be varied over a wide range by changing the concentration of actin. An estimate for the rate constant of exchange can then be obtained by relating these turnover times to measured values for oxygen exchange (incorporation of ^{18}O from $H_2{}^{18}O$ into the inorganic phosphate (P_i) released by hydrolysis). The results of such an experiment, with turnover times between 0.2 and 25 s, indicate that, for each γ-phosphoryl group, one oxygen from the medium is added rapidly (to cleave the phosphoryl group or form a pentacoordinate phosphoryl complex); two more oxygens exchange with a rate constant, k_e, of about 1 s^{-1}; and a fourth oxygen exchanges slowly with k_e about 0.2 s^{-1}. The higher value is about 18 times smaller than the rate constant, k_{-3}, for the reverse cleavage step of the myosin pathway, which is postulated to be responsible for oxygen exchange. The data, then, indicate that the rate-limiting step for oxygen exchange is not k_{-3}, but may be the rate of rotation of oxygens around the phosphorus atom, with one oxygen severely restricted by its binding to the active site. The finding that k_e differs for the four oxygens in each phosphate group is related to past observations on myosin-catalyzed oxygen exchange.

During the turnover of a molecule of MgATP at the active site of myosin, there is an extensive exchange of oxygen between water and the terminal phosphate group of the nucleotide. The exchange was discovered during experiments on myosin ATPase using an ^{18}O label to follow the transfer of oxygen (Levy and Koshland, 1958, 1959). It was evident from the earliest studies of the reaction that it occurred at an intermediate step in the hydrolysis of ATP by the contractile proteins, myosin and actomyosin (Levy and Koshland, 1959; Levy et al., 1960, 1962) and that the exchanging intermediate was probably an important part of the mechanism of muscle contraction (Levy and Koshland, 1959; Yount and Koshland, 1963).

A significant advance toward understanding oxygen exchange was made in 1966 by Sartorelli et al. who described the properties of a myosin–ATP complex which had exchanged oxygen and appeared to have undergone cleavage of the terminal phosphate bond. Then, most recently, Bagshaw et al. (1975) substantially sharpened our view of the exchange mechanism by showing that two intermediates, M*·ATP and M**·ADP·P_i,[1] now known to occur during ATP hydrolysis by myosin or its subfragments, are directly involved in oxygen exchange. The current pathway of myosin hydrolysis (Bagshaw and Trentham, 1973, 1974; Baghsaw et al., 1974, 1975) has these two intermediates occurring in Scheme I. M, M*, and M** represent different conformations of myosin distinguished by fluorescence. Step 3, the transformation of M*·ATP to M**·ADP·P_i, is called the cleavage step. In simplest terms it is the step in which the covalent terminal phosphate bond of the bound ATP is broken. However, on the basis of current knowledge, it could also represent the transformation of a pentavalent phosphate intermediate to a more labile form which subsequently cleaves (Young et al., 1974). The rate-limiting step in the sequence is step 4 which for myosin acting alone is exceedingly slow (Bagshaw and Trentham, 1974; Bagshaw et al., 1974). As we will discuss more fully later, actin activation of myosin hydrolysis is believed to be the result of the activation of this step (Lymn and Taylor, 1971).

SCHEME I

$$\mathrm{M} + \mathrm{ATP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \mathrm{M}\cdot\mathrm{ATP} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftarrows}} \mathrm{M}^{*}\cdot\mathrm{ATP} \underset{k_{-3}}{\overset{k_{+3}}{\rightleftarrows}} \mathrm{M}^{**}\cdot\mathrm{ADP}\cdot\mathrm{P_i} \underset{k_{-4}}{\overset{k_{+4}}{\rightleftarrows}}$$

$$\mathrm{M}^{*}\cdot\mathrm{ADP}\cdot\mathrm{P_i} \underset{k_{-5}}{\overset{k_{+5}}{\rightleftarrows}} \mathrm{M}^{*}\cdot\mathrm{ADP} + \mathrm{P_i} \underset{k_{-6}}{\overset{k_{+6}}{\rightleftarrows}} \mathrm{M}\cdot\mathrm{ADP} \underset{k_{-7}}{\overset{k_{+7}}{\rightleftarrows}} \mathrm{M} + \mathrm{ADP}$$

The finding by Bagshaw and Trentham (1973) that the reverse cleavage step, k_{-3}, is relatively rapid compared with the overall rate of hydrolysis led them to suggest that oxygen exchange occurs because the cleavage reaction adds an atom of oxygen to the phosphoryl group and the reverse takes one away. Both k_{+3} and k_{-3} (≥ 160 and ≥ 18 s^{-1}, respectively) are fast enough to repeat many times before the turnover of one molecule of ATP (Bagshaw and Trentham, 1974; Bagshaw et al., 1974; Lymn and Taylor, 1970). If the oxygen lost by the reverse reaction were different from the one gained by cleavage, one atom of oxygen would exchange during each cycle. For the oxygen atom lost to be different from the one gained requires only that the oxygens around the phosphorus rotate in space. If this rotation were not rate limiting, then the rate-limiting step for oxygen exchange would be simply k_{-3}. On the other hand, a restriction of rotation could limit the exchange rate of any or all the oxygens around the phosphorus atom.

The purpose of this paper is to present the results of an ex-

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[1] Abbreviations used: P_i, inorganic phosphate; Nbs_2, 5,5'-dithiobis(2-nitrobenzoate); Tris, tris(hydroxymethyl)aminomethane; HMM, heavy meromyosin; 2,4-DNP, 2,4-dinitrophenol; S-1, subfragment 1; ATP, adenosine triphosphate; ITP, inosine triphosphate.

periment designed to choose between these and other alternatives by estimating the apparent rate constant of oxygen exchange. To do this we took advantage of the fact that actin, depending on its concentration, can increase the rate of ATP hydrolysis by subfragment 1 (S1) up to 100 times or more (Eisenberg et al., 1968). Kinetic studies, for the most part, indicate that it does this by greatly speeding up step 4 without directly modifying the cleavage reaction (Lymn and Taylor, 1971), through the formation of an actin–M**·ADP·P_i complex. Thus, actin effectively removes M**·ADP·P_i from the cleavage cycle. If we assume that reverse cleavage is the cause of oxygen exchange (Bagshaw and Trentham, 1973; Boyer et al., 1973) and that actin activates hydrolysis at step 4 (Lymn and Taylor, 1971), then the effect of actin is to "quench" the exchange reaction. This natural quenching by actin gives us the chance to compare oxygen exchange at different turnover times without changing any other condition of the experiment. Depending only on the concentration of actin, the average time available for exchange can be varied over a wide range, the oxygen exchange occurring in this time can be measured, and the apparent rate constant for exchange can be estimated.

Experimental Procedure

Protein Preparations. Rabbit back and leg muscles were used for all the protein preparations. Actin was prepared from acetone-dried muscle powder by the method of Spudich and Watt (1971). Subfragment 1 (S1) was prepared from myosin by the method of Margossian et al. (1975) with Mg^{2+} present to preserve the Nbs_2 light chain. The last step involving ammonium sulfate fractionation was omitted. The electrophoretic pattern of this S1 on a 10% polyacrylamide gel was identical with that of Margossian et al. (1975) for what they call Mg–S1.

Hydrolysis of ATP in $H_2{}^{18}O$. Reaction mixture was prepared in $H_2{}^{18}O$ containing 25 mM KCl, 25 mM Tris buffer (pH 7.4), 5 mM $MgCl_2$, and appropriate amounts of S1 and actin at 23 °C. At each actin concentration, the amount of S1 was adjusted so that theoretical 100% hydrolysis would occur at about the same time (45–90 min). The reaction was started by adding ATP to a final concentration of 5 mM. Periodically, 0.5-ml aliquots of the reaction mixture were taken to assay for inorganic phosphate (Koshland and Clarke, 1953). These assays were used for determining the rate of ATP hydrolysis. At 80 to 100% hydrolysis, the reaction was quenched by adding trichloroacetic acid to a final concentration of 10%. The acidifed reaction mixture was treated with acid-washed charcoal (10 mg per μmol of nucleotide) for 30 min at 0 °C, to remove nucleotides. The denatured protein and charcoal were then removed by vacuum filtration through a small Büchner funnel. 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.

Isolation of P_i and Conversion to CO_2 to Obtain ^{18}O Content. The P_i was precipitated as $MgNH_4PO_4$, dried with alcohol and ether, and ultimately isolated as KH_2PO_4 essentially by the method of Dempsey et al. (1963). KH_2PO_4 (15 mg) was converted to CO_2 by heating with 0.1 g of guanidine hydrochloride (Mann) for 8 h at 250 °C in a sealed vial according to the method of Boyer and Bryan (1967). This CO_2 sample was then analyzed for its 46/(44 + 45) ratio on the mass spectrometer. The ^{18}O content of the medium water was determined in an identical fashion by heating 5 μl of the medium water with 0.1 g of guanidine hydrochloride.

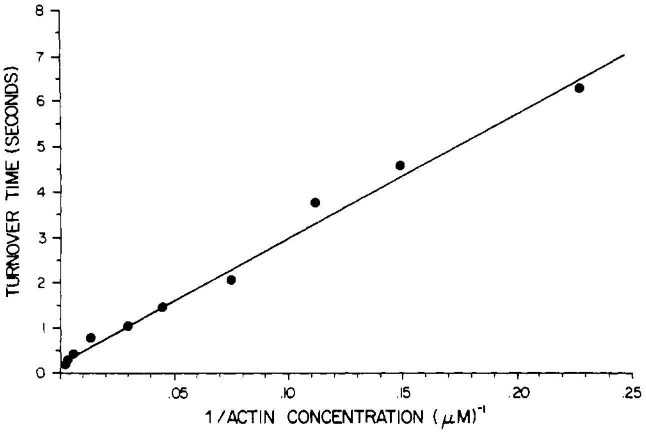

FIGURE 1: Dependence of turnover time on actin concentration. Conditions: 5 mM ATP, 5 mM $MgCl_2$, 25 mM Tris buffer (pH 7.4), and 25 mM KCl. The molecular weight of S1 was taken as 120 000, and the molecular weight of actin as 45 000.

Results

Effect of Actin on Turnover Time. Figure 1 shows the effect of actin concentration on the turnover time for ATP hydrolysis by S1. At each concentration of actin, the rate of hydrolysis was obtained from the slope of a linear rate curve and converted to a turnover number (molecules of ATP hydrolyzed per second per active site). The turnover time on the ordinate is the reciprocal of this number. Thus, Figure 1 is a double-reciprocal plot of the kind used by Eisenberg et al. (1968) to estimate the apparent binding constant of actin to S1 and the apparent V_{max} for actin-activated S1 hydrolysis. The exact interpretation of the line given by such a plot, in terms of the kinetics of hydrolysis, is not clear at this time (Moos, 1972), but for our purposes here this does not matter.

Under the conditions of these experiments, the apparent affinity of actin was exceedingly weak. Even at the highest levels of actin we could use (when the reaction mixtures were actually thick gels), the rates did not appear to be close to a plateau. Nevertheless, at the highest concentration of actin (440 μM), the turnover of 5 s^{-1} was 125 times greater than the turnover for S1 alone. This rate is matched only by the fast initial "burst" rate of actomyosin gel; the steady-state rate of actomyosin gel is only about 2 s^{-1}.

Effect of Actin on Oxygen Exchange. Table I shows the incorporation of ^{18}O from water into the P_i released by hydrolysis. The measured values for ^{18}O incorporation do not take into account those exchange reactions between ^{18}O in the water and ^{18}O already incorporated into a phosphate group. To estimate the actual number of exchanges it is necessary to correct mathematically for such recycling of the label. A general approach to this, which makes no specific assumptions about mechanism, gives a value for exchange expressed as the product of k_e (the rate constant for exchange) and t (the lifetime of the exchanging intermediate) (Levy and Koshland, 1959):

$$k_e t = \ln\,[0.75/(1 - X)] \tag{1}$$

where X = fraction of oxygen-18 incorporated. The values in column 5 of Table I were calculated using the alternate equation of Wolcott and Boyer (1975):

$$\frac{\text{water oxygens}}{P_i\ \text{molecule}} = \sum_{n=1}^{n} (3/4)^{n-1} \tag{2}$$

where n = the number of exchange reactions per P_i molecule.

TABLE I: Effect of Actin Concentration on the Turnover Time of ATP Hydrolysis by Subfragment 1 and the Extent of Oxygen Exchange.

Actin Concn) (mg/ml)	Turnover Time (s)	No. of ^{18}O Atoms Incorp per Phosphate	$k_e t^a$	No. of Exchange Reactions per Phosphate, n^b
0	25	3.78	2.6	>9
0.2	6.3	3.65	2.2	8.5
0.3	4.6	3.71	2.3	8.7
0.4	3.8	3.54	1.9	7.6
0.6	2.1	3.34	1.5	6.3
1.0	1.5	3.33	1.5	6.2
1.5	1.0	2.78	0.90	4.2
3.0	0.75	2.69	0.83	3.9
3.3	0.75	2.74	0.87	4.0
7.8	0.41	2.19	0.51	2.8
16.5	0.28	2.04	0.43	2.4
20.3	0.19	1.60	0.22	1.7

[a] See eq 1 in the text. [b] See eq 2 in the text.

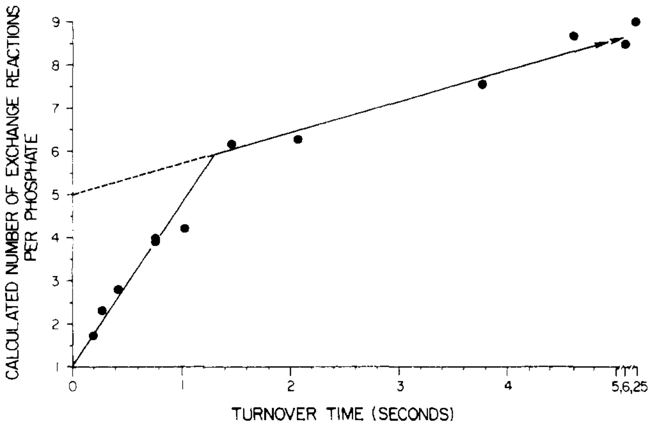

FIGURE 2: Calculated number of exchange reactions per phosphate group vs. turnover time. Conditions: see Figure 1.

This equation gives the number of oxygen exchanges per phosphate group assuming that the cleavage cycle is the mechanism of exchange. The two estimates for the exchange are approximately related by the following equation:

$$1 + 3k_e t \cong n \tag{3}$$

Because $k_e t$ in eq 1 is a continuous function of ^{18}O incorporation, while n in eq 2 occurs in discrete steps, the two estimates can be expected to differ by a few percent.

The Incorporation of ^{18}Oxygen as a Function of Turnover Time. Figure 2 shows how the calculated number of exchange reactions per phosphate group changed with the turnover time (set by the actin concentration). It is clear from this figure that not all the oxygen atoms exchanged at the same apparent rate. Instead the data fit two lines, indicating a rate of exchange of about 4 reactions phosphate^{-1} s^{-1} during the first second of turnover time (the initial steep slope) but less than 0.8 reaction phosphate^{-1} s^{-1} for the remaining time (the shallow upper slope). The steep line intersects the ordinate at a value of 1, indicating a very rapid exchange of the first oxygen. This is consistent with the idea that the first cleavage reaction adds an oxygen at a rate equal to k_{+3} ($\geq$160 s^{-1}). A curve analogous to that of Figure 2 was obtained by plotting the calculated values of $k_e t$ against the turnover time. The two slopes of this curve directly gave the apparent values for k_e: about 1.2 s^{-1} for the fast exchange (which corresponds with the 4 cycles s^{-1} of Figure 2); and 0.2 s^{-1} for the slow exchange (which corresponds with the slower exchange of 0.8 cycle s^{-1} of Figure 2).

Discussion

We interpret these results in the following way:

(1) The lines drawn in Figure 2 are functions of different rates of exchange for the four oxygens in a phosphate group: (a) an exceedingly fast addition of the first ^{18}O by the cleavage reaction, k_{+3} ($\geq$160 s^{-1}), (b) a relatively fast exchange of two oxygens, and (c) a slow exchange of one oxygen.

(2) After 1 s or so, virtually all the faster exchanging oxygens are labeled. From that point on, nearly all the incorporation of label is into the one slowly exchanging oxygen. Thus, the shallow slope of Figure 2 is an estimate of the slow exchange rate; while the steeper slope is the sum of the fast and slow rates. Consistent with this assumption, the shallow line extrapolates back to a value of 5 exchanges/phosphate, the number required to label three of the four oxygens.

(3) The apparent rate constant, k_e, for the fast exchange is about 1 s^{-1} and for the slow exchange about 0.2 s^{-1}. The apparent k_e within a turnover time of about 1 s is the sum of these two constants, about 1.2 s^{-1}. These esimates were obtained from the slopes of a plot of $k_e t$ vs. t, as described in the Results section.

(4) The average rate constant for exchange would be greater than 5 s^{-1} (18 exchanges phosphate^{-1} s^{-1} $\leq k_{-3}$) if it were limited by the reverse cleavage reaction. But the estimate of k_e from our data is only 1 s^{-1} for the faster exchange.

(5) Since k_{-3} does not appear to be rate limiting, it seems reasonable to assume that some restriction of rotation around the phosphorus limits the rate of exchange. If so, then such restriction is particularly severe for one oxygen in each phosphoryl group.

(6) This slowly exchanging oxygen may be bound to the protein. It seems reasonable to assume this, especially if the bound nucleotide in the M**·ADP·P$_i$ is cleaved. As pointed out by Young et al. (1974), an ease of rotation of all the oxygens in a bound phosphate group contradicts the relatively tight binding of the P$_i$ in this complex.

(7) For a bound oxygen to exchange, it would have to dissociate and change places with another oxygen. In a sense, this is what happens in what is called medium exchange (Dempsey et al., 1963; Swanson and Yount, 1966), when the phosphate group is actually released into the medium to mix with the inorganic phosphate pool; then a P$_i$ together with a molecule of ADP reforms the exchanging intermediate by a reversal of steps 7 through 4 in the hydrolytic pathway. Given sufficient time, such medium exchange would completely label the oxygen since it gives all four oxygens in a phosphate group the chance to come into an exchanging (unbound) position at the active site. But medium exchange is too slow under the conditions of these experiments to account for the extensive labeling of the fourth oxygen (Levy et al., 1962, Swanson and Yount, 1966, Wolcott and Boyer, 1975). There are, however, other plausible mechanisms. The bound oxygen could exchange by occasionally coming loose and rotating without the phosphate group necessarily being released into the medium. Since such "loosening" of the bound oxygen need not occur too frequently to account for the observed exchange, such a mechanism is consistent with the reversal of a step involving sub-

stantial energy loss. We will refer to this kind of exchange as "slow exchange" to distinguish it from both the faster intermediate exchange and medium exchange.

(8) Past measurements of oxygen exchange catalyzed by myosin, heavy meromyosin (HMM), and S1 are consistent with the hypothesis that only three of the four oxygens per phosphate readily exchange. But the restriction of exchange of the fourth oxygen is not absolute. Even for myosin, where values for exchange come closest to three oxygens/phosphate (exchange with myosin subfragments is generally higher), the average from a large number of measurements (over 20) in our laboratory is closer to 3.2. The actual values are quite variable in these unpublished, as well as in published, data and can be as high as 3.6. Such variations are most probably not experimental error but the reason for them is unknown. It is interesting, in this regard, to note that the modifier, 2,4 dinitrophenol (2,4-DNP), significantly increases the extent of oxygen exchange catalyzed by myosin, up to values as high as 3.8 oxygens/phosphate (Koshland and Levy, 1964; Sartorelli et al., 1966), suggesting that other modifications in the protein by, e.g., age, preparative procedures, or experimental conditions, may alter the tightness of binding of the phosphoryl group in the exchanging intermediate and, thus, affect slow exchange of the bound oxygen.

(9) The work reported here, and past measurement of oxygen exchange, are, for the most part, consistent with the kinetic data which indicate that factors such as the presence of actin, or the substitution of ITP for ATP, primarily affect k_{+4} (the rate-limiting step in the hydrolytic pathway) and not the cleavage step. In general, the reason why oxygen exchange and overall rate of hydrolysis have, in the past, failed to show any simple relationship is probably related to the following factors which have become apparent in the course of the work reported here: (a) rapid intermediate exchange is over after 1 s or so. Therefore, large percentage variations in the turnover time for hydrolysis between 1 and 25 s show comparatively little effect on exchange. (b) Few, if any, modifiers other than actin can reduce the turnover time sufficiently to have any substantial effect on the rapid exchange. (c) Even a small direct effect on the slow and variable exchange of the fourth oxygen could mask the effect of a large change in turnover time. For example, 2,4-DNP which, as noted, can increase myosin exchange from about 3 to as high as 3.8 oxygens/phosphate, increases the rate of hydrolysis sevenfold (Levy et al., 1963). Yet this large change in rate, which reduces the turnover time from 25 to 4 s, would cause no noticeable decrease in the fast exchange. Thus only an increase in the slow exchange would be apparent.

(10) The results are consistent with the earlier finding that actomyosin gel in the steady state with a turnover time of 0.5 s shows an incorporation of 2.0-2.3 atoms of labeled oxygen per phosphate (Levy and Koshland, 1959; Koshland and Levy, 1964, and unpublished data); in the burst phase, with an average turnover time of about 0.25 s, actomyosin showed an incorporation of 1.9 atoms of labeled oxygen (unpublished data). The curve of Figure 2 is also in agreement with the reported values for ^{18}O incorporation into $M^{*}\cdot ATP$ and $M^{**}\cdot ADP\cdot P_i$. The incorporation into these complexes of 3 atoms per γ-phosphoryl group would be expected from the exchange time of 2 s set by acid quenching in the experiments of Bagshaw et al. (1975).

(11) The effect of calcium stands out as a significant exception to the general rule that many modifiers and conditions affect overall hydrolysis at step 4 without directly affecting the cleavage step. Calcium, when it replaces magnesium at the ATPase site, completely prevents oxygen exchange during hydrolysis (just as it completely prevents superprecipitation of actomyosin gel and muscle contraction). This cannot be explained by the effect of calcium on turnover time. The turnover time of CaATPase is of the order of 0.5 s, comparable to the MgATPase of actomyosin gel or S1 activated by an actin concentration of 7 mg/ml (Table I). In this time, with magnesium, there is still substantial oxygen exchange (Figure 2). This strongly indicates that the pathway for myosin hydrolysis with calcium at the active site is profoundly different from the mechanism with magnesium.

(12) The results presented here are consistent with either of the two current ideas about the nature of the exchanging intermediate: (a) that $M^{*}\cdot ATP$ contains a bound form of uncleaved nucleotide and that $M^{**}\cdot ADP\cdot P_i$ is a high-energy form of cleaved nucleotide, having a phosphate moiety tightly bound to the protein (Bagshaw and Trentham, 1973); (b) that both $M^{*}\cdot ATP$ and $M^{**}\cdot ADP\cdot P_i$ are pentacoordinate phosphoryl intermediates formed by the addition of water to bound ATP (Young et al., 1974). Whatever the exact chemical mechanism for step 3, the estimates for the rate constants of oxygen exchange presented here support the idea that rotation, not the reverse cleavage step (k_{-3}), limits the exchange rate of three oxygens in the phosphoryl group and that protein binding severely restricts the exchange of the last one.

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Dynamic Reversal of Enzyme Carboxyl Group Phosphorylation as the Basis of the Oxygen Exchange Catalyzed by Sarcoplasmic Reticulum Adenosine Triphosphatase[†]

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ABSTRACT: Millisecond mixing and quenching experiments demonstrate an apparent $t_{1/2}$ for the labeling of phosphorylated sarcoplasmic reticulum ATPase by $^{32}P_i$ at pH 6 and 30 °C of 30 to 40 ms. Under the same conditions, the rate of exchange of water oxygens with inorganic phosphate (P_i) is about 40 mol of H_2O exchanged with P_i per 10^6 g of protein per s. Theoretical equations are developed for the expected ^{32}P-labeling pattern given various comparative rates of flux between P_i and the Michaelis complex and between the Michaelis complex and phosphorylated enzyme. The results show that the rapid reversal of the formation of the phosphorylated enzyme is a major source of the oxygen exchange and are consistent with such reversal being the only source.

The sarcoplasmic reticulum ATPase of skeletal muscle has been shown to catalyze a rapid exchange between oxygens of inorganic phosphate and water (a $P_i{}^1 \rightleftharpoons$ HOH exchange) in the presence of Mg^{2+} (Kanazawa and Boyer, 1973). The cleavage of ATP by the enzyme proceeds with intermediate formation of a phosphoenzyme (see Hasselbach, 1974) and a phosphoenzyme can be formed from P_i in the presence of Mg^{2+} and absence of added adenine nucleotides or a Ca^{2+} gradient (Kanazawa and Boyer, 1973; Masuda and de Meis, 1973; Kanazawa, 1975; de Meis, 1976). The phosphoryl group has been demonstrated to be attached to the carboxyl group of the side chain of an aspartic acid residue (Degani and Boyer, 1973) and is formed by displacement of a water oxygen from P_i (Dahms et al., 1973). These findings suggested that the rapid $P_i \rightleftharpoons$ HOH exchange may result from dynamic reversal of the formation of the phosphoenzyme from enzyme and P_i.

The purpose of the studies reported herein was to use rapid mixing and quenching techniques to measure the rate of formation and cleavage of the phosphoenzyme to find if this reaction may account for the oxygen exchange. Such measurements contribute to the understanding of the catalysis by the ATPase and are relevant to the mechanism of other enzyme-catalyzed oxygen exchanges.

Experimental Procedures

Phosphoenzyme Formation and Measurement. The phosphorylation of the sarcoplasmic reticulum ATPase was conducted with membrane vesicle preparations from rabbit skeletal muscle under conditions and with materials as described elsewhere (Masuda and de Meis, 1973; de Meis, 1976). The experiments were designed to measure the rate of interchange between P_i and the phosphoenzyme (E–P) under steady-state conditions. This has the advantage that any possible changes in rates of reaction steps accompanying the initial exposure of the enzyme to P_i are avoided. Thus the enzyme preparation was incubated with unlabeled P_i for about 10 min before filling of the syringes for the rapid mixing. The reaction mixture containing enzyme was then mixed rapidly with an equal volume of a solution of identical composition but without enzyme and with $^{32}P_i$ (approximately 10^8 cpm per ml) present, followed by quenching upon mixing with an approximately equal volume of 0.89 M perchloric acid. Control samples for measurement of total E–P were incubated with $^{32}P_i$ for 20 s, a period quite sufficient for maximal formation of phosphoenzyme under the reaction conditions. The amount of ^{32}P-labeled phosphoenzyme in the quenched samples was measured as previously described (Masuda and de Meis, 1973).

The P_i concentration used (6 mM) was approximately three times the concentration required for half-maximal E–P for-

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[1] Abbreviations used: P_i, inorganic phosphate; E–P, phosphoenzyme.