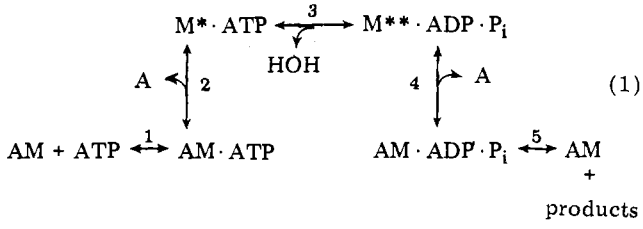
Effect of Actin Concentration on the Intermediate Oxygen Exchange of Myosin; Relation to the Refractory State and the Mechanism of Exchange†

J. A. Sleep and Paul D. Boyer*

ABSTRACT: The effect of actin concentration on the myosin catalyzed exchange of phosphate oxygens with water accompanying ATP hydrolysis has been investigated. The extent of exchange was found to extrapolate to zero at infinite actin concentration at 23 and 0 °C for myosin subfragments S1(A1) and S1(A2). This result is consistent with actin associating directly with the product of the hydrolysis step and is not readily consistent with refractory state schemes in which the entire flow goes via a dissociating pathway. The possibility of a refractory state in the form of a phosphorylated intermediate or a bound metaphosphate state with hydrolysis occurring in the transition to the refractory state merits consideration. A full analysis of the dependence of intermediate exchange on the rate constants of the acto-S1 scheme is given and the errors arising from other methods of analysis are discussed. The rate of oxygen exchange was measured as 10 s^{-1} (23 °C) a value comparable with but slightly lower than the rate of reversal of the ATP cleavage step.

Lymn & Taylor (1971) have proposed a kinetic scheme of actomyosin ATPase (eq 1), the central features of which are generally accepted.



(1)

This scheme was initially interpreted with the assumption that all the steps were irreversible except for step 4 and that step 5 was rate limiting at saturating actin concentration. On this basis the scheme predicted the observed hyperbolic dependence of the steady-state ATPase rate on actin concentration (Eisenberg & Moos, 1968) with $V_m = k_5$ and $K_m = (k_{-4} + k_5)/k_4$. The steady-state rate is thus $k_5A/(K_m + A)$.

In a series of papers (Eisenberg & Kielley, 1972; Fraser et al., 1975; Chock et al., 1976), Eisenberg and co-workers have investigated the degree of association and the steady-state rate under conditions of low ionic strength at temperatures in the range 0 to 15 °C. They found, using a variety of techniques, that, at actin concentrations sufficiently high to yield steady-state rates near V_{max}, 50–90% of the S1[1] was still dissociated from actin, an observation inconsistent with the interpretation of the Lymn–Taylor scheme outlined above. This finding implies that steps in the dissociated part of the scheme contribute to rate limitation.

In the scheme of eq 1 the only state which could exist at saturating actin concentrations is M*·ATP which would necessitate hydrolysis (step 3) being the rate-limiting step. Until recently the rate of the hydrolysis step had not been measured under the low ionic strength conditions which were used in the acto-S1 experiments of Eisenberg and collaborators, and at higher ionic strengths this rate did not correspond well to the V_m for acto-S1. The existence of a new myosin product state, the refractory state, was proposed to account for these observations (eq 2):

(2)

In this scheme the transition from the refractory, M**·ADP·P_i(R), to the nonrefractory state, M**·ADP·P_i(NR), is the rate limiting step at saturating actin concentrations.

Two factors need consideration when interpreting these data. More recent measurements show that the rate of the ATP cleavage step (Johnson & Taylor, 1978) is ionic strength dependent and that the value at low ionic strength is closer to the V_{max} of acto-S1 than previously thought. The need to introduce a new myosin product state as a refractory state is correspondingly less, for M*·ATP partly fills this role. The second point relates to the irreversibility of the steps of the scheme. Lymn (1974a,b) made a more detailed analysis of the Lymn–Taylor scheme; he noted that the hyperbolic dependence of the steady-state rate on actin concentration arose because only one state of the myosin scheme, M**·ADP·P_i, was being titrated by actin in the range of experimentally accessible actin concentrations. Current evidence on the deviation from a hyperbolic dependence (Marston, 1978) suggests that actin does begin to titrate the state M*·ATP at high actin concentrations which means that V_{max} would be less than the rate of any first order step in the scheme. This also helps the state M*·ATP to assume the role of the refractory state but the point remains that introduction of a refractory state does improve the fit to the data, particularly at pH 8 (E. Eisenberg, personal communication).

Determination of the correct explanation of the low degree of association of actin and S1 during ATP hydrolysis at actin concentrations which give rates close to V_m is of importance

†From the Department of Chemistry and the Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024. *Received May 23, 1978.* This work was done during the tenure of a research fellowship (to J.A.S.) of the Muscular Dystrophy Association. This work was supported by Grant No. PCM 75-18884 from the National Science Foundation.

[1] Abbreviations used: S1, myosin subfragment 1; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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not only for the understanding of the enzyme mechanism but also for models of the crossbridge cycle in muscle (Eisenberg & Hill, 1978). As developed in the present paper, observation of the dependence of intermediate $P_i \rightleftharpoons HOH$ exchange upon actin concentration offers a useful approach to determining whether a refractory state exists and, if so, to measurement of the rate constants leading to and from this state.

Current evidence is consistent with oxygen exchange phenomena arising from reversal of the hydrolytic cleavage step of the ATPase pathway (Bagshaw et al., 1975). Assuming that the rate of P_i rotation does not limit the rate of exchange (a point which will be considered later), the extent of exchange depends on the number of reversals of the hydrolysis step, which is governed by the relative rates of reversal and leaving the exchange step. If the rate of leaving the exchange step is much greater than the rate of reversal, there will be no reversals and no exchange. In the Lymn–Taylor scheme, actin has direct access to the product of the hydrolytic step and the rate of departure from this step is linearly dependent on actin concentration and thus oxygen exchange should extrapolate to zero at infinite actin concentration. A crucial distinguishing feature of a scheme including a refractory state is that actin would lack this direct access. The maximum rate of leaving the intermediate exchange step would be close to V_m, the steady-state rate at a saturating actin concentration. This rate, together with the rate of reversal of the ATP cleavage step, would thus set a limit on the minimum amount of exchange.

Shukla & Levy (1977a–c) have also measured the extent of intermediate exchange as a function of actin concentration. They concluded that the P_i oxygens are nonequivalent with respect to exchange, three exchanging fast and the fourth exchanging five times more slowly. This is a plausible interpretation of the data but it has been shown by more direct methods involving the use of highly enriched [γ-^{18}O]ATP and the formation of a volatile derivative of the product P_i for direct mass spectral analysis (Sleep et al., 1978), that the P_i oxygens are equivalent[2] and that apparent nonequivalence arises from the contribution of a second ATPase pathway to the overall flux. In addition, Shukla & Levy (1977a) from a limited analysis of their data concluded that the maximal rate of intermediate oxygen exchange was slower than expected if the exchange were limited by the rate of reversal of the hydrolytic cleavage step. A more complete analysis given in this paper shows that the discrepancy is much smaller than suggested by Shukla & Levy, although still experimentally significant.

Experimental Section

Proteins. Myosin was prepared by the method of Perry (1955) from the back and hind leg muscles of rabbit. Myosin subfragment 1 was made by chymotryptic digestion and separated into the A1 and A2 fractions as described by Weeds & Taylor (1975). Actin was prepared by the method of Drabikowski & Gergely (1964). The pellets were resuspended as F-actin in the appropriate buffer using a glass homogenizer. Actin was shown to be free of tropomyosin and troponin, and the A1 and A2 fractions of S1 were shown to have the appropriate light chain content by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Protein concentrations were determined by absorption at 280 nm using the following extinction coefficients, 0.77 cm^2/mg for S1 (Young et al., 1965) and 1.09 cm^2/mg for actin (Rees & Young, 1967). The molecular weights used were 115 000 for S1 (Lowey et al., 1969) and 42 000 for actin (Elzinga et al., 1973). The reaction mixtures consisted of reaction buffer, ^{18}O-enriched water, actin, S1, and pyruvate kinase, the reaction being initiated by the addition of MgATP (1:1) and phosphoenolpyruvate neutralized to pH 7 with Tris. The S1 had been dialyzed against and the actin homogenized with reaction buffer.

Analyses. Reaction mixtures were quenched with an equal volume of 1 M perchloric acid, 5 mM EDTA at a time when about 80% of the ATP and phosphoenolpyruvate had been hydrolyzed. Activated charcoal (100 mg) was added to absorb nucleotides and the charcoal and protein precipitate were removed by centrifugation. The P_i was isolated and the ^{18}O content determined essentially as described by Boyer & Bryan (1967). The ^{18}O content of the water in the reaction mix was determined by equilibration with CO_2 and analysis with a ratio mass spectrometer (Weber et al., 1974). Steady-state rates of ATP hydrolysis were determined by spectrophotometric measurement of NADH disappearance in a coupled assay using pyruvate kinase and lactate dehydrogenase.

Interpretation of Exchange Measurements. Intermediate $P_i \rightleftharpoons HOH$ exchange arises from the reversal of a step in which oxygen from medium water becomes covalently bound to phosphorus. The current evidence (Bagshaw et al., 1975) is consistent with the relevant step being that of the hydrolytic cleavage of ATP (eq 3) and the problem will be treated on this basis. The derivation is equally applicable to other possible mechanisms, involving pentacoordinate intermediates (Young et al., 1974), metaphosphate (Sartorelli et al., 1966), or phosphorylated protein intermediates. This general problem has been examined by Boyer et al. (1977) but the outline is given here explicitly for the case of intermediate exchange. The *O/P* ratio, defined as the number of atoms of oxygen derived from water present in the product, P_i, is given by eq 4

$$M + ATP \longrightarrow M^{**} \cdot ATP \underset{k_-}{\overset{HOH}{\rightleftharpoons}} M^{**} \cdot ADP \cdot P_i \xrightarrow{k_+} \quad (3)$$

$$O/P \text{ ratio} = \sum_0^\infty p(n) O/P(n) \quad (4)$$

where $p(n)$ is the probability of n reversals before the release of P_i, and $O/P(n)$ is the average *O/P* ratio of P_i released after n reversals.

If all four oxygens are equivalent and the rate of exchange is controlled by the rate of reversal, the chance of a water oxygen incorporated into bound P_i being retained on reformation of ATP and HOH is 3/4. If there are n reversals prior to release of P_i, then one oxygen is incorporated into the product from the final, $(n + 1)$th, cleavage, 3/4 from the nth, and $(3/4)^{n+1-i}$ from the ith. Thus the net extent of exchange of water oxygens with P_i oxygens for n reversals is $\Sigma_{i=0}^{n} (3/4)^i$ as given in Wolcott & Boyer (1975). The probability that P_i will be released from $M^{**}\cdot ADP\cdot P_i$ without further reversals to $M^{*}\cdot ATP$ is $y = k_+/(k_- + k_+)$ (eq 3: k_+ represents the forward flux), while the probability of reversal is $(1 - y)$. The probability of n reversals prior to leaving is thus $p(n) = y(1 - y)^n$. The infinite series (eq 4) for the *O/P* ratio has an analytic solution given by eq 5.

$$O/P = 4/(1 + 3y) \quad (5)$$

The extent of intermediate exchange depends on the ratio of the rate of reversal from $M^{**}\cdot ADP\cdot P_i$ to $M^{*}\cdot ATP$ and the flux leaving the $M^{**}\cdot ADP\cdot P_i$ state in the direction of product release.

[2] The distribution for acto-S1 intermediate exchange, at actin concentrations which give *O/P* ratios in the range 2 to 3, fits the theoretical distribution for equivalent oxygens very well (J. A. Sleep, D. D. Hackney, & P. D. Boyer, manuscript in preparation).

$$\rightarrow M^{*}\cdot ATP \underset{HOH}{\overset{3}{\rightleftharpoons}} M^{**}\cdot ADP\cdot P_i \underset{A}{\overset{4}{\rightleftharpoons}} AM\cdot ADP\cdot P_i \quad \downarrow 5 \quad (6)$$

$$AM + \text{products}$$

$$\text{flux} = k_4[A] - \frac{k_{-4}[AM\cdot ADP\cdot P_i]}{[M^{**}\cdot ADP\cdot P_i]}$$

$$\frac{d[AM\cdot ADP\cdot P_i]}{dt} = k_4[A][M^{**}\cdot ADP\cdot P_i] - (k_{-4} + k_5)[AM\cdot ADP\cdot P_i]$$

$$= 0 \text{ during the steady state}$$

$$\text{flux} = k_5k_4[A]/(k_{-4} + k_5) \quad (7)$$

The flux is thus linearly dependent upon actin concentration, and the value to be assigned to $k_5k_4/(k_{-4} + k_5)$ is dependent upon the scheme. At low actin concentrations the rate-limiting step is actin binding to M**·ADP·P_i and thus the term $k_5k_4/(k_{-4} + k_5)$ is simply related to V_m/K_m. For the scheme in eq 1, $k_4k_5/(k_{-4} + k_5) = (V_m/K_m)(1 + K_3)/K_3$ and the value of K_3 is obtained from experiments of the type described by Bagshaw & Trentham (1973).

We will consider a refractory state model in which all the rate limitation comes from the hydrolysis and refractory to nonrefractory state steps and in which $K_{3b} \lesssim 1$ (eq 8).

$$\begin{array}{ccc} & & M + \text{products} \\ & & \uparrow \\ \rightarrow M^{*}\cdot ATP & \overset{3a}{\underset{HOH}{\rightleftharpoons}} & M^{**}\cdot ADP\cdot P_i(R) \\ & & \updownarrow 3b \\ & & M^{**}\cdot ADP\cdot P_i(NR) \\ & & A \downarrow 4 \\ \text{products} + AM & \overset{5}{\leftarrow} & AM\cdot ADP\cdot P_i \end{array} \quad (8)$$

There are several reasons for making the latter assumption but the most relevant one for the present purposes is that, if step 3b were irreversible, the flux from the refractory state would be k_{3b} at all actin concentrations and the *O/P* ratio would correspondingly be independent of actin concentration. The assumption that $K_{3b} \lesssim 1$ maximizes the actin dependence of the intermediate exchange and is thus the most favorable case to consider. In the following derivation [R] and [NR] represent the concentrations of the refractory and nonrefractory states.

$$\text{flux} = k_{3b} - k_{-3b}[NR]/[R]$$

$$\frac{d[NR]}{dt} = k_{3b}[R] - (k_{-3b} + k_4[A])[NR]$$

$$[NR]/[R] = k_{3b}/(k_{-3b} + k_4[A])$$

$$\text{flux} = k_{3b}\left(1 - \frac{1}{(1 + k_4[A]/k_{-3b})}\right) \quad (9)$$

The flux must be related to the steady-state parameters V_m and K_m via the known values of k_{3a} and k_{-3a}.

At low actin concentrations step 4 is the rate-limiting step and all the myosin is in the form of dissociated states which are in equilibrium.

$$V_m/K_m = k_4K_{3a}K_{3b}/(1 + K_{3a} + K_{3a}K_{3b})$$

If $K_{3b} \lesssim 1$

$$V_m/K_m \approx k_4K_{3a}K_{3b}/(1 + K_{3a})$$

$$\frac{k_4}{k_{-3b}} = \frac{V_m}{K_m}\frac{(1 + K_{3a})}{K_{3a}k_{3b}} \quad (10)$$

At saturating actin concentration $V_m = k_{3b} \times$ (fraction of myosin existing as the refractory state)

$$\frac{d[R]}{dt} = k_{3a}[M^{*}\cdot ATP] - (k_{-3a} + k_{3b})[R]$$

$$[M^{*}\cdot ATP] = [R](k_{-3a} + k_{3b})/k_{3a}$$

$$\frac{[R]}{[R] + [M^{*}\cdot ATP]} = k_{3a}/(k_{3a} + k_{-3a} + k_{3b})$$

$$V_m = k_{3b}k_{3a}/(k_{3a} + k_{-3a} + k_{3b})$$

$$k_{3b} = V_m(k_{3a} + k_{-3a})/(k_{3a} - V_m) \quad (11)$$

continuing from eq 9 and using eq 10 and 11

$$\text{flux} = \frac{V_m(k_{3a} + k_{-3a})}{(k_{3a} - V_m)} \times \left[1 - \frac{1}{\left(1 + \frac{[A](1 + K_{3a})(k_{3a} - V_m)}{K_mK_{3a}(k_{3a} + k_{-3a})}\right)}\right] \quad (12)$$

The total flux from M**·ADP·P_i is the sum of the flux due to interaction with actin and the flux for S1 in the absence of actin. This is analogous to the fact that one plots $1/(V - V_s)$ vs. $1/A$ in order to get linear plots of the acto-S1 steady-state rate as a function of actin concentration (Eisenberg & Moos, 1968), V_s being the steady-state rate of ATP hydrolysis for S1 alone. At 23 °C an adequate measure for the contribution of S1 is simply $V_s(1 + K_3)/K_3$. At 0 °C, due to the existence of two rate-limiting steps for S1 ATPase which are approximately equal (Bagshaw & Trentham, 1974), one must double this number.

Results

The extent of intermediate exchange as a function of actin concentration is shown in Figures 1-3 for S1(A1) at 23 °C, S1(A2) at 23 °C, and S1(A1) at 0 °C, respectively. In each case the *O/P* ratio starts from a value approaching the limit of 4 (complete exchange) in the absence of actin and extrapolates to a value of 1.0 (no exchange) at infinite actin concentration. The general behavior of the curve is as expected; with increase in actin concentration the time available for exchange and thus the average number of reversals and the *O/P* ratio all decrease. The *O/P* ratio for S1 in the absence of actin is somewhat variable from preparation to preparation in the range 3.7 to 3.86 at 23 °C and in the range 3.3 to 3.5 at 0 °C.

The corresponding values for V_m and K_m for the steady-state ATPase rates were 13.6 s^{-1} and 16 μM (Figure 1), 22.6 s^{-1} and 32 μM (Figure 2), and 0.3 s^{-1} and 18 μM (Figure 3).

Figures 1-3 show that the theoretical curves (solid lines) derived from a simple Lymn-Taylor scheme (eq 1) in which actin binds directly to the product of the exchange step (hydrolytic cleavage of ATP) fit the data well at moderate and high actin concentrations; both theoretical and experimental curves extrapolate to an *O/P* ratio of 1.0 at infinite actin concentration. The theoretical curves are based upon rates of reversal of 10 and 12 s^{-1} for S1(A1) and S1(A2) at 23 °C and 0.5 s^{-1} for S1(A1) at 0 °C. These rates are in reasonably good agreement with measurements of the apparent rate of reversal by medium exchange (Sleep et al., 1978) which gave rates of 15 s^{-1} (S1(A2), 25 °C) and 0.67 s^{-1} (S1(A2), 0 °C). A recent

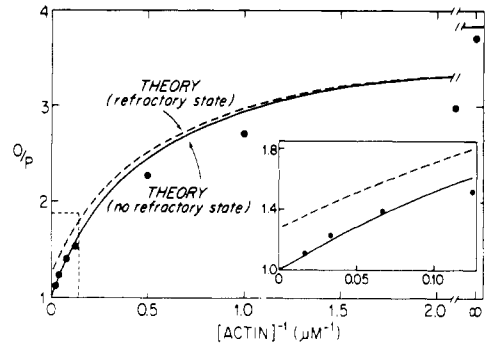

FIGURE 1: Effect of actin concentration on the intermediate $P_i \rightleftharpoons HOH$ exchange of acto-S1(A1). Theoretical curve (—) for a simple Lymn-Taylor model with $V_m = 13\ s^{-1}$; $K_m = 16\ \mu M$; $K_3 = 2.2$; $k_{-3} = 10\ s^{-1}$. Theoretical curve (— —) for a refractory state model with $(k_{3a} + k_{-3a}) = 80\ s^{-1}$ (Johnson & Taylor, personal communication). Experimental conditions: 23 °C, 3 mL of 5 mM KCl, 1 mM $MgCl_2$, 10 mM Mes-Tris (pH 7), 0.5 mM MgATP, 1.75 mM phosphoenolpyruvate, 200 μg of pyruvate kinase, the specific actin concentration and concentrations of S1 to give similar overall hydrolysis times (2 h). The insert is an enlargement of that region of the graph enclosed by the dotted line.

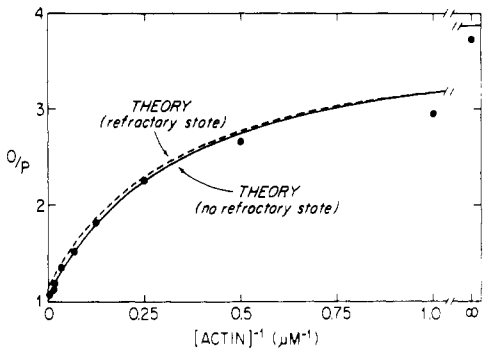

FIGURE 2: As Figure 1 but for S1(A2). Theoretical curves based on the following values, $V_m = 23\ s^{-1}$, $K_m = 32\ \mu M$, $K_3 = 2.2$; $k_{-3} = 12\ s^{-1}$; $(k_{3a} + k_{-3a}) = 80\ s^{-1}$.

measurement of acto-S1 intermediate exchange, in which the analysis was done by the volatile phosphate method, gave a rate of 16 s^{-1}. These values are lower limits for the true rates of reversal because it is possible that exchange is limited by other features, for example, rotation of the bound P_i. The apparent rates of reversal derived by exchange methods are somewhat lower than the rates determined from measurements of K_3 and k_3. At 23 °C the equilibrium constant K_3 is 2.2 (Sleep & Hutton, 1978) and $(k_3 + k_{-3})$ is 80 s^{-1} (K. Johnson & E. Taylor, University of Chicago, personal communication) which give a value for k_{-3} of 25 s^{-1}. At 0 °C a similar estimate would be about 2 s^{-1} and at this temperature the discrepancy would appear to be significant. As has already been pointed out limitation by the rate of rotation of P_i or accessibility of water are possible explanations for this discrepancy. Another possible explanation which could be of more consequence is that ATP cleavage and hydrolysis are separate steps and we will consider this possibility in more detail later. A small contribution to this discrepancy may arise because the rate of reversal determined from $(k_3 + k_{-3})$ and K_3 is dependent on direct measurement of first-order rates which are unaffected by any inactive enzyme. The measurement derived from oxygen exchange involves the use of the steady-state ATPase rate which is affected.

The dashed curves in Figures 1-3 are theoretical curves for a refractory state model in which rate limitation at saturating actin concentrations is solely due to the refractory to nonre-

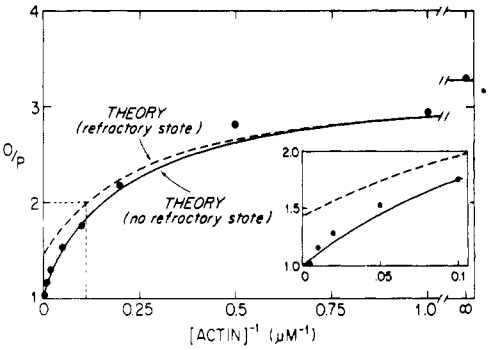

FIGURE 3: As Figure 1 but at 0 °C, S1(A1), 3 mL of 10 mM KCl, 1 mM $MgCl_2$, 1 mM MgATP, 3 mM phosphoenolpyruvate. Theoretical curves based on the following values: $V_m = 0.3\ s^{-1}$, $K_m = 18\ \mu M$, $K_3 = 1$, $k_{-3} = 0.5\ s^{-1}$, $(k_{3a} + k_{-3a}) = 4\ s^{-1}$. The insert is an enlargement of that region of the graph enclosed by the dotted line.

fractory step and the hydrolysis step. The refractory state curve for S1(A1) at 0 °C (Figure 3) extrapolates to an O/P ratio of 1.43 at infinite actin concentration in contrast to the experimental extrapolation to 1.0. The amount that the extrapolated value is above 1.0 for a refractory state model depends primarily on the ratio of V_{max} to the apparent rate of reversal measured by intermediate exchange. This ratio is larger at 23 °C than at 0 °C and the theoretical extrapolated value (1.26) is closer to 1.0 (Figure 1). S1(A2) has a higher V_{max} than S1(A1) and thus the extrapolated value (1.15) becomes still closer to 1.0 (Figure 2).

It is important to emphasize why the discrepancy in the value extrapolated to infinite actin is significant, when the discrepancy between experiment and theory at low actin concentrations is of a comparable size. When the O/P ratio is close to 1.0, it becomes very insensitive to small changes in k_+ (eq 3) and thus to change the extrapolated value from 1.05 to 1.43 (S1(A1), 0 °C) means changing the value of k_+ by a factor of 10. This general type of experiment has been repeated many times under a variety of conditions for both S1 and HMM. The extrapolated values have always been less than 1.1 and are generally in the range 1.00 to 1.05. The original evidence in favor of a refractory state was obtained at 0 °C and it is at this temperature that the infinite actin value for the refractory state model (O/P = 1.43) differs most markedly from the experimental value. The discrepancy at 23 °C, while still outside the limits of experimental error, is comparatively small (A1, 1.26; A2, 1.15). We thus conclude that a refractory state model in which all the rate limitation occurs in the dissociated part of the scheme is not consistent with the data. The simple Lymn-Taylor scheme is consistent with the oxygen exchange data, although it does not fully account for the data of Eisenberg and collaborators or the results of Sleep & Hutton (1978).

Our data are interpreted on the assumption that an experimentally important kinetic ^{18}O-isotope effect on the O/P ratio is absent. In a symposium presentation, Wolcott & Boyer (1975) reported preliminary data suggesting that the O/P ratio for S1 CaATPase was less than 1.0, reflecting a possible kinetic isotope effect. If this were correct, an isotope effect might occur for MgATPase and the acto-S1 intermediate exchange would extrapolate to less than 1.0 at infinite actin concentration. As we do not observe such an extrapolated value, the intermediate exchange of S1 CaATPase was measured in triplicate using internal $[^{18}O]P_i$ standards to check for P_i contamination during the analysis. We observed an O/P ratio of 1.025 at pH 8, 50 mM KCl, and 25 °C, conditions under which the S1 CaATPase is close to maximal, and a value of 1.03, at pH 7, 10

mM KCl and 25 °C, the conditions under which the acto-S1 experiments were carried out. Thus the previous report suggesting a kinetic isotope effect is not substantiated.

Discussion

The principal objective of this work was to clarify the status of the refractory state, but before this is discussed it is necessary to consider the mechanism of intermediate exchange and the equivalence of the bound P_i oxygens, a question emphasized by the work of Shukla & Levy (1977a-c). They interpreted their data in terms of the bound P_i oxygens being nonequivalent with respect to exchange, 3 oxygens exchanging rapidly and the fourth more slowly. This was a plausible suggestion; however, methods of following oxygen exchange in which P_i oxygens are converted to CO_2 can suggest nonequivalence but not prove it. Sleep et al. (1978) have approached this question by the use of highly ^{18}O-labeled ATP or P_i with determination of the distribution of product phosphate species with 0, 1, 2, 3, and 4 ^{18}O oxygens. They have shown that for S1 medium exchange the P_i oxygens behave in an equivalent manner with respect to exchange and that for intermediate exchange myosin has a lower O/P ratio than S1 due to myosin preparations catalyzing a second pathway of ATP hydrolysis with no intermediate exchange. The observed distribution was quite different from that predicted on the basis of a nonequivalent model of the type suggested by Shukla & Levy.[2]

The theoretical curves given in this paper are based on an equivalent model and it can be seen that the fit is only fair at low actin concentrations. It is likely that this apparent nonequivalence is due to a much reduced contribution of the second pathway for S1 preparations. Conclusions relating to the existence of the refractory state depend on the value of the O/P ratio extrapolated to infinite actin concentration, which is not affected by the experimental points at low actin concentration, and for this reason no corrections have been made to the theoretical curves.

As we have seen the data are not consistent with a refractory state model in which all the rate limitation at saturating actin concentration is due to the hydrolysis and refractory to nonrefractory state steps. One cannot conclude that a state M**·ADP·P_i(R) does not exist but one can conclude that the contribution to rate limitation of the step from refractory to nonrefractory states must be so small that the state no longer fulfills the role for which it was introduced: to provide a rate-limiting step between dissociated states.

There appears to be one way in which this conclusion can be avoided: if ATP cleavage and hydrolysis do not take place in the same step but M*·ATP is cleaved to M**·Pr(R) and hydrolyzed to M**·ADP·P_i(NR), then exchange would take place by reversal of the refractory to nonrefractory state step. Actin would bind to M**·ADP·P_i(NR) which is the product of the exchange step and thus intermediate exchange would decrease to zero at infinite actin concentration as is observed. In such a scheme the state M**·Pr(R) would have to be a phosphorylated intermediate or a metaphosphate state. None of the experiments which have been performed in search of such states (Sartorelli et al., 1966; Trentham, 1977) have produced any evidence in their favor but the experiments were not of the type which enable the existence of such states to be ruled out. For this reason we feel a scheme in which cleavage and hydrolysis are separate steps is worthy of attention despite the lack of direct evidence in its favor.

The possible contribution of a nondissociating pathway

$$\mathrm{AM \cdot ATP} \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} \mathrm{AM \cdot ADP \cdot P_i} \xrightarrow{k_5}$$

to the total ATPase flux is relevant when considering the consistency of the refractory state with oxygen exchange data. The contribution will increase with actin concentration and, if significant, this pathway must be associated with an O/P ratio of 1.0. If it were to become dominant at accessible actin concentrations, then the O/P ratio would extrapolate to 1.0 at infinite actin concentration even with a refractory state model: this provides a second way in which such a model could be consistent with the oxygen exchange data.

Sleep & Homsher (unpublished observations) have measured the intermediate exchange of both isometric glycerinated rabbit muscle fibers and of myofibrils using single turnover techniques to avoid overcontraction. Both methods give an O/P ratio of about 2 which suggests that the nondissociating pathway may not make a major contribution to the total ATPase flux in muscle.

Shukla & Levy's interpretation of their data differs in important ways from ours aside from questions of equivalence of the phosphate oxygens, and as their conclusions have quite general significance it seems desirable to be explicit as to why our interpretations differ. They conclude that the rate of the step limiting exchange is 1 s^{-1} (Shukla & Levy, 1977a), which is an order of magnitude slower than the rate of reversal of the cleavage step and thus they are forced to postulate that the rate of rotation is rate limiting. Using our method to analyze their data, we obtain a value of about 20 s^{-1} for the rate of exchange, which is close to the rate of reversal of the cleavage step determined by other kinetic methods. As the difference in conclusions is of considerable importance we will consider the cause in detail.

The first point is that Shukla & Levy (1977a) gave a rate constant for exchange, k_c, of about 1 s^{-1}, whereas in their Figure 2 the number of reversals for a turnover time of 1 s is about 4 and thus the rate of reversal must be at least 4 s^{-1}. This error is to some extent corrected in their third paper (Shukla & Levy, 1977c).

A second point relates to the use by Shukla & Levy of the turnover time as the time available for exchange. We deal with the problem in terms of competing rates and show that the flux from M**·ADP·P_i is linearly dependent on actin concentration for a nonrefractory state model. This is equivalent to saying that the time available for exchange goes to zero at infinite actin concentration. In contrast the turnover time reaches a minimum corresponding to V_m at saturating actin concentration. The error in using the turnover time is large if actin concentrations greatly above the K_m are used. The K_m for actin of actomyosin ATPase is much lower than for acto-S1 (Burke et al., 1974) and thus at moderate actin concentrations complete saturation is achieved. This would appear to be the major reason why Shukla & Levy (1977c) concluded that the rate of exchange of myosin differed greatly from that of S1. Use of turnover time also gives a relatively small error at all actin concentrations due to the omission of a term of the type $(1 + K_3)/K_3$.

A third point relates primarily to the question of equivalence but has some repercussions for the rate. At any given actin concentration there is a distribution of the number of reversals of the cleavage step and the experimentally observed O/P ratio is the result of an average of this distribution. In our theoretical treatment, we perform the averaging in eq 4 ($O/P = \Sigma_0^\infty p(n)O/P(n)$); we are averaging the product of the probability of n reversals and the average O/P ratio for this number of reversals, a procedure not open to doubt. Shukla & Levy plot n, the number of reversals needed on the basis of the equation of Wolcott & Boyer (1975) $O/P(n) = \Sigma_{i=0}^{n} (3/4)^i$, against the average turnover time. By expecting their plot to be a straight

line for equivalent oxygens, they are in effect taking the product of the average number of reversals and the average *O/P* ratio for this number of reversals. This method of averaging will give the correct result under only a limited set of conditions, namely, if $p(n)$ is symmetrical about the average value of n, and $O/P(n)$ is linearly dependent on n. Neither of these conditions are met and thus one would not expect their plot to be linear even if all four oxygens were equivalent.

Finally a comment about possible cooperativity between myosin heads (Shukla & Levy, 1977b) may be helpful. The intermediate exchange studies of actin activated myosin and its proteolytic subfragments may reveal interesting differences but before firm conclusions can be drawn it is necessary to analyze the distribution of labeled P_i species (Sleep et al., 1978; Webb et al., 1978) to ensure that the product phosphate is derived from a single pathway and to analyze the experimental data in an appropriate manner.

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