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Analysis of Positional Isotope Exchange in ATP by Cleavage of the βP–OγP Bond. Demonstration of Negligible Positional Isotope Exchange by Myosin†

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ABSTRACT: A method for analysis of positional isotope exchange (PIX) during ATP ⇌ HOH oxygen exchange is presented that uses a two-step degradation of ATP resulting in cleavage of the βP–OγP bond. This cleavage yields P_i derived from the γ-phosphoryl of ATP that contains all four of the γ oxygens. Both PIX between the β,γ-bridge and β-nonbridge positions and washout of the γ-nonbridge oxygens can be simultaneously followed by using ATP labeled with ^{17}O at the β-nonbridge positions and ^{18}O at the β,γ-bridge and γ-nonbridge positions. Application of this method to ATP ⇌ HOH exchange during single turnovers of myosin indicates that the bulk of the ATP undergoes rapid washout of γ-nonbridge oxygens in the virtual absence of PIX. At 25 °C with subfragment 1 the scrambling rate is at the limit of detectability of approximately 0.001 s^{-1}, which is 50-fold slower than the steady-state rate. This corresponds to a probability of scrambling for the β-oxygens of bound ADP of 1 in 10000 for each cycle of reversible hydrolysis of bound ATP. A fraction of the ATP, however, does not undergo rapid washout. With myosin and stoichiometric ATP at 0 °C, this fraction corresponds to 10% of the ATP remaining at 36 s, or 2% of the initial ATP, and an equivalent level of ATP is found that does not bind irreversibly to myosin in a cold chase experiment. A significant level of apparent PIX is observed with subfragment 1 in the fraction that resists washout, and this apparent PIX is shown to be due to contaminant adenylate kinase activity. This apparent PIX due to adenylate kinase provides a possible explanation for the PIX observed by Geeves et al. [Geeves, M. A., Webb, M. R., Midelfort, C. F., & Trentham, D. R. (1980) *Biochemistry 19*, 4748–4754] with subfragment 1.

Positional isotope exchange (PIX)[1] during a reaction provides information on the generation of intermediates that possess rotational freedom [see Rose (1979)]. Thus, cleavage of ATP at the βPO–γP bond leaves the β,γ-bridge oxygen with the ADP, and torsional movement will result in scrambling of this original bridge oxygen with the two original β-nonbridge oxygens. Re-formation of ATP can then occur with incorporation of an original β-nonbridge oxygen into the bridge position. This PIX can be detected by using ATP labeled with

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[1] Abbreviations: PIX, positional isotope exchange; PRPP, 5-phospho-D-ribosyl 1-pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; CaATPase, calcium adenosinetriphosphatase activity of myosin; DAPP, P^1,P^5-di(adenosine-5′) pentaphosphate; S1, myosin subfragment 1; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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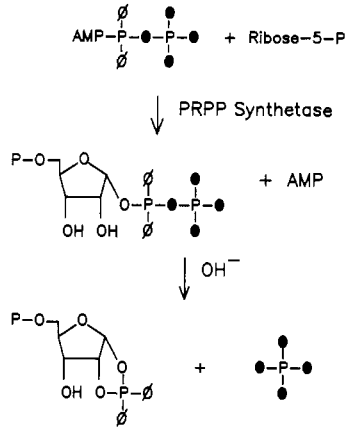
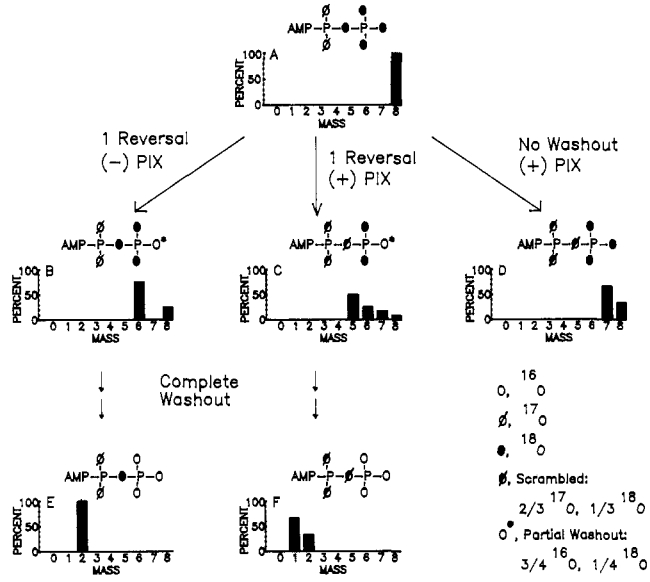
Scheme I



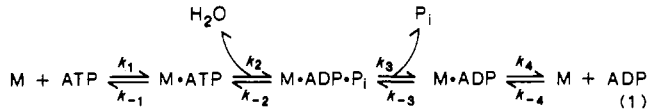
Scheme II



^{18}O or ^{17}O in either the β-bridge or β-nonbridge position with exchange shifting part of the label into the other class of oxygens. The position of isotopic labeling can be determined by ^{31}P NMR by either the ^{18}O-induced shift in the chemical shift or by the line broadening induced by ^{17}O [see Cohn (1982) and Reynolds et al. (1983)]. The NMR approach has the advantages that degradation is not required and reactions can be monitored in real time; but it requires very large samples for analysis, and this makes it impractical for single turnover reactions and many other cases where only small samples are available. Alternatively, the position of isotopic substitution can be determined by mass spectroscopy following specific degradation of the ATP. Previous work employing mass spectroscopy has been limited by the fact that selective enzymatic cleavage of ATP occurs by addition of a nucleophile to the γ-phosphoryl with retention of the β,γ-bridge oxygen in the ADP. Thus, it is not possible to directly separate the β,γ-bridge oxygen from the β-nonbridge oxygens so that the isotopic content of each pool can be determined. One approach to circumvent this problem involves scrambling the β- and γ-positions [see Midelfort and Rose (1976) and Webb (1980)], so that on subsequent cleavage half of the P_i will contain the β-nonbridge oxygens.

We now report a degradation scheme that produces selective cleavage of the βP–OγP bond yielding P_i derived from the γ-phosphoryl of the ATP containing the β,γ-bridge oxygen in addition to the three γ-nonbridge oxygens. This method involves the intermediate information of PRPP followed by its nonenzymic decomposition in base as indicated in Scheme I. This decomposition of PRPP was originally reported by Khorana et al. (1958), and we demonstrate here that the β,γ-bridge oxygen is released quantitatively with the P_i. Since this P_i contains all four γ-oxygens, it is possible to *simultaneously* follow both PIX and washout of the γ-nonbridge oxygens during ATP $\rightleftharpoons$ HOH exchange on the same ATP sample. This is accomplished with ATP labeled at all four γ-oxygens with ^{18}O and with the β-nonbridge oxygens labeled with ^{17}O as indicated in Scheme II for the idealized case of 100% isotopic labeling. Washout of γ-nonbridge oxygens by ATP $\rightleftharpoons$ HOH exchange in the absence of PIX results in the species 8 mass units above the mass of the unenriched species being converted sequentially into species at +6, +4, and ultimately +2 mass units. Occurrence of PIX will result in the formation of species at odd mass unit intervals above the mass of the unenriched species due to introduction of the ^{17}O-labeled β-nonbridge oxygens into the β,γ-bridge position.

The myosin ATPase reaction occurs with extensive washout of the oxygens originally in the γ-nonbridge position, and this exchange has been proposed to be due to the reversible hydrolysis of the ATP while bound at the active site as indicated in eq 1 (Bagshaw et al., 1975; Webb & Trentham, 1981b).



Mixing of myosin with a stoichiometric amount of ATP results in essentially irreversible binding of ATP in a two-step process summarized in step 1, followed by numerous cycles of reversible hydrolysis of the bound ATP in step 2 before rate-limiting product release occurs. Geeves et al. (1980) have reported that the ATP isolated from M·ATP during such a single turnover has undergone a variable amount of PIX, averaging 45%, and they interpret this to indicate that enzyme-bound ADP is being reversibly generated and has significant, although not complete, torsional freedom. Using the method of Scheme I, we report here an analysis of PIX by myosin in the presence of DAPP, a potent inhibitor of adenylate kinase (Lienhard & Secemski, 1973), which indicates that most of the bound ATP undergoes rapid washout of the γ-nonbridge oxygens with an almost complete lack of PIX. A second pool of ATP is also observed that has not undergone significant washout. This pool of un-washed-out ATP is greatly reduced, but not eliminated, when myosin binding sites are in excess of the ATP level. In the absence of DAPP, significant total PIX is observed in the un-washed-out ATP, and the change in mass distribution is that expected for ATP formed by adenylate kinase from the [β-^{17}O]ADP released by myosin. This apparent PIX catalyzed by adenylate kinase provides a possible explanation for the PIX observed by Geeves et al. (1980).

Materials and Methods

Rabbit skeletal muscle myosin and myosin subfragment 1 (papain EDTA S1) were made essentially according to the method of Margossian and Lowey (1982). S1 was concentrated by ammonium sulfate fractionation, and myosin was purified by chromatography on DEAE-Sephadex by the method of Offer et al. (1973). PRPP synthetase was isolated from *Salmonella typhimurium* by the method of Switzer and Gibson (1978) using a hisG mutant (hisG46 kindly provided by Bruice Ames) to eliminate possible contamination with ATP phosphoribosyl transferase. The PRPP synthetase was dialyzed exhaustively against 0.1 M sodium arsenate, pH 8, before use. All other enzymes were obtained from Sigma Chemical

Co. Labeled phosphate was made by the procedure of Hackney et al. (1980) from 55% [^{17}O]water and from 90% [^{18}O]water (Cambridge Isotope Labs). [γ-^{32}P]ATP and [^{18}O]PEP were prepared by the method of Hackney and Clark (1985), and [β-^{32}P]ADP was synthesized from [γ-^{32}P]ATP by reaction with adenylate kinase and excess AMP and purified by ion-exchange chromatography. Dowex-1 (AG1-X4) was obtained from Bio-Rad Laboratories.

[β-^{17}O,γ-^{18}O]ATP. An initial batch of [β-^{17}O]ADP was made from AMP morpholidate and [^{17}O]P_i essentially by the method of Wehrli et al. (1965). This [β-^{17}O]ADP was then used to initiate the coupled enzymatic synthesis of additional [β-^{17}O]ADP via reaction with glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, glyceraldehyde 3-phosphate, and [^{17}O]P_i to transiently generate [β,γ-^{17}O]ATP, which was trapped by transfer of its γ-phosphoryl to excess AMP by adenylate kinase. The [β-^{17}O]ADP was purified on DEAE-cellulose by elution with a linear gradient of triethylammonium bicarbonate, converted to [β-^{17}O,γ-^{18}O]ATP by condensation with [^{18}O]P_i by the method of Wehrli et al. (1965), and purified on DEAE-cellulose. This procedure involves intermediate synthesis of ADP morpholidate and results in all four γ-oxygens being derived from the [^{18}O]P_i.

PRPP Synthetase Control Experiments. Several isotopically labeled ATP samples were prepared to test the usefulness of the PRPP synthetase method of analysis. These samples were prepared as described below and were analyzed according to Scheme I to obtain P_i, which is derived from the γ-phosphoryl and contains the β,γ-bridge oxygen.

Completely scrambled, unexchanged ATP was obtained by incubation of 6 mM MgATP for 11 h with 0.1 mg/mL creatine phosphokinase and 30 mM creatine in 65 mM Tris, pH 8.0, 10 mM KCl, and 1 mM DTT at 25 °C. Control experiments with [γ-^{32}P]ATP indicated that half-equilibration of creatine phosphate formation from [^{32}P]ATP occurred in 3 min.

Phosphate containing the three γ-nonbridge oxygens and one water-derived oxygen was obtained by hydrolysis of ATP with 0.2 μM chymotryptic S1 (Margossian & Lowey, 1982) in pH 8 buffer containing 35 mM Tris, 30 mM KCl, 2 mM $CaCl_2$, 0.1 mM EDTA, and 0.1 mM DTT. The ADP obtained from this hydrolysis contains the β,γ-bridge oxygen, and it was rephosphorylated to ATP by using PEP and pyruvate kinase to simulate complete PIX and a degree of washout of the γ-nonbridge oxygens controlled by the ^{18}O-enrichment of the PEP. Unlabeled PEP was used to obtain scrambled ATP that was unlabeled in the three γ-oxygens; [^{18}O]PEP, which was at a lower enrichment than the starting ATP, was used to obtain ATP that appeared to have undergone complete scrambling with partial washout of the γ-oxygens.

PIX Analysis. PIX reactions were conducted either at 0 °C in 15 mM Aces [2-[(carbamoylmethyl)amino]ethanesulfonic acid], 15 mM Mops [3-(*N*-morpholino)propanesulfonic acid], 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 mM DTT at pH 7.0 or at 25 °C in 30 mM KCl, 35 mM Tris-HCl, 2 mM $MgCl_2$, 0.1 mM EGTA, and 0.1 mM DTT at pH 8.0. The reaction was initiated by addition of labeled ATP to a rapidly stirred suspension of myosin or S1 that had been dialyzed against the selected buffer. The reactions were stopped by addition of cold concentrated perchloric acid to a final concentration of 0.5 M. The precipitated protein was removed by centrifugation, and the pellet was washed to increase the recovery of ATP. The combined supernatants were neutralized with KOH and centrifuged to remove precipitated $KClO_4$, and the pellet was washed to remove adsorbed ATP. The residual dissolved perchlorate was removed by passage at pH 2 through a column containing excess Dowex-1 followed by elution with 0.5 M HCl to remove any bound ATP. The combined eluents were neutralized, diluted to low ionic strength, loaded onto a 1.6-mL column of Dowex-1, washed with 10 mM HCl, and eluted with 30, 100, and 500 mM HCl to remove P_i, ADP, and ATP, respectively. The ATP-containing fractions were neutralized, diluted to low ionic strength, and rechromatographed on a smaller 0.3-mL column of Dowex-1 as above. The peak ATP fractions in 500 mM HCl were pooled, Tris was added to 100 mM, and the pH was adjusted to 8 by addition of KOH.

For reaction with PRPP synthetase, the neutralized ATP fraction was adjusted to 100 mM arsenate, 4 mM ribose 5-phosphate, 4 mM $MgCl_2$, 0.1 mM EDTA, and 0.1 mM DTT. (PRPP synthetase requires either P_i or arsenate for activation, and arsenate was chosen to avoid unwanted sources of P_i.) The reaction was initiated at 36 °C by addition of sufficient PRPP synthetase to obtain 95% completion in 10–20 min. The reaction was stopped by vigorous mixing with chloroform, nucleotides were removed with charcoal, and the supernatant was diluted and loaded onto a 1-mL DEAE-cellulose column. The column was washed with 50 mM KCl to remove arsenate, and the PRPP was eluted with 200 mM KCl. The PRPP was decomposed by treatment with 50 mM $BaCl_2$ and 4 mM KOH for 1 h at 25 °C. The reaction mixture was diluted, loaded onto a 0.5-mL Dowex-1 column and washed with 10 mM HCl, and the P_i was eluted with 30 mM HCl.

Very large amounts of myosin were required because of the single turnover nature of the reactions and because the equilibrium of step 2 favors formation of ADP and P_i and loss of bound ATP. Thus for series 5 of Table II at 25 °C with a very unfavorable value of K_2, reaction of 21.1 μmol (2.3 g) of S1 with 14.9 μmol ATP yielded 0.7 μmol of P_i following isolation of the reacted ATP and degradation via Scheme I. The other series with more favorable values of K_2 required use of less myosin.

Mass Spectroscopic Analysis. Mass spectroscopic analysis was performed on triethyl phosphate obtained by reaction with diazoethane (Stemple & Boyer, 1986). Most of the phosphate samples were analyzed by electron impact ionization on a Hewlett-Packard Model 5992 gas chromatograph/mass spectrometer. The isotopic cluster from *m/e* 99 to 107 was used, with *m/e* 99 corresponding to fully ^{16}O-labeled $P(OH)_4^+$. Unenriched P_i yields almost exclusively *m/e* 99 in the region of 93–106 except for minor fragments of 2.2, 0.5, 0.55, 0.08, and 0.5% at *m/e* 93–97, respectively, and the contribution from natural abundance of ^{2}H, ^{17}O, and ^{18}O at *m/e* 100 and 101. The peak at *m/e* 93 contains three oxygens as indicated by a peak of corresponding size at *m/e* 99 in the mass spectrum of 99% triethyl [^{18}O]phosphate (no corresponding peak is seen at *m/e* 155, indicating that the peak at *m/e* 99 is not due to contamination with unenriched P_i). Correction was made for this fragment by assuming that it contained three oxygens. No correction was necessary for the fragment at *m/e* 94 as it contains less than three oxygens, with no peak observed at *m/e* 100 or 102 with 99% ^{18}O-enriched P_i. Unambiguous assignment of the oxygen content of the other minor fragments is not possible because of their small, similar sizes, but corrections were applied for masses 95 and 96 as three-oxygen fragments. It was also found that the line shape of the peaks was asymmetric with 0.2–0.6 and 0% spillover of the *m/e* 99 peak into the observed *m/e* 98 and 100, respectively, and this was corrected for by sequential subtraction of this factor from the next lower mass peak starting with *m/e* 107. Unenriched

Table I: Characterization of [β-^{17}O,γ-^{18}O]ATP[a]

sample		distribution mass above all ^{16}O species (%)								
		0	1	2	3	4	5	6	7	8
1	starting [^{17}O]P_i	0.22	0.26	1.92	8.34	20.0	31.9	25.0	10.7	1.88
	theory, 53% ^{17}O, 36.9% ^{18}O	0.01	0.22	1.87	8.41	21.3	30.7	25.0	10.7	1.85
2	starting [^{18}O]P_i	0	0.02	0.26	0.01	3.97	0.35	27.9	0.97	66.5
	theory, 90.3% ^{18}O, 0.33% ^{17}O	0.01	0	0.30	0.03	4.28	0.30	27.6	0.97	66.6
3	γ-P_i, CaATPase	*b*	0.33	3.38	0.33	24.5	0.84	70.6		
	theory, 89.3% ^{18}O, 0.33% ^{17}O	0.11	0.01	2.88	0.18	24.8	0.79	71.2		
4	initial ATP	0.36	0.23	0.65	0.14	4.42	0.36	30.7	0.92	62.2
	theory	0.01	0	0.40	0.04	5.15	0.33	29.5	0.94	63.6
5	scrambled	*b*	0.34	1.48	0.94	7.85	6.41	30.5	18.9	33.3
	theory	0.03	0.03	0.82	0.80	8.01	6.69	31.2	18.7	33.9
6	scrambled + [^{18}O]PEP	0.50	0.75	4.17	2.81	13.2	10.2	30.9	13.5	23.7
	theory	0.64	0.62	3.64	2.42	14.3	9.49	31.2	13.3	24.5
7	scrambled + [^{16}O]PEP[c]	26.8	24.5	48.1	0.09	0.1	0.21	0.1	0.04	0.22
	theory	26.6	25.8	47.6	0	0	0	0	0	0

[a] Sample 3 was obtained by S1 CaATPase cleavage of the γ-P_i of untreated ATP. Samples 4–7 were obtained by the analysis indicated in Scheme I. Theoretical distributions calculated for 89.3% ^{18}O and 0.33% ^{17}O in the γ-nonbridge and β,γ-bridge oxygens and for 26.8% ^{18}O and 38.5% ^{17}O in the β-nonbridge oxygens. The distribution of the [^{18}O]PEP used for sample 6 is 2.4%, 9.4%, 36.8%, and 51.4% for masses +0, +2, +4, and +6, respectively. [b] Corrected for contamination with unenriched P_i of 3.7% and 1.2% in samples 3 and 5. These observed amounts for the +0 mass were set to zero, and the remaining distribution was renormalized. [c] Corrected for natural abundance by subtracting 0.14% and 0.82% of mass +0 from mass +1 and mass +2, respectively, and adding these back to mass +0.

P_i also contains a fragment of 0.6% at *m/e* 107 that was corrected for when applicable. The response of the mass detector was not strictly uniform across the 99–107 mass range, and this was corrected for by using the mass spectrum of a standard mixture of unenriched P_i and 99% [^{18}O]P_i whose composition was confirmed by ^{31}P NMR. These corrections varied slightly with tuning of the mass spectrometer and were redetermined with each set of analyses. The resulting corrected distributions are expressed as the percentage of the P_i having masses at 0–8 mass units above the mass for 100% [^{16}O]P_i. Some of the samples were analyzed on a Varian MAT112 gas chromatograph/mass spectrometer that exhibited a similar fragmentation pattern.

It should be noted that significantly larger corrections would be required with other procedures for determining the true mass distribution. Both analysis of the *m/e* cluster at 155 for electron impact of triethyl phosphate and chemical ionization of trimethyl phosphate have the complication of the large natural abundance of ^{13}C at +1 mass, and both have significant fragmentation into the −1 and −2 masses.

ADP Dissociation Rate. The rate of ADP dissociation from S1 was measured at 0 °C by the change in intrinsic fluorescence of S1 induced by displacement of ADP by PP_i (Sleep et al., 1981). The fluorescence was measured by using λ_{ex} = 295 nm and λ_{em} = 340 nm, and the reaction was conducted in the same buffer used for the PIX measurements. ADP was initially present at 50 μM and $MgPP_i$ added to 4 mM, and the first-order rate constant was determined for the resulting decrease in fluorescence.

Equivalence Point Titrations. Steady-state titrations were done in the PIX buffer by the method of Hackney and Clark (1985). S1 was titrated with MgATP in the presence of 0.5 mg/mL pyruvate kinase and 1 mM PEP to regenerate ATP. The ATPase rate was measured by coupling the regenerating system with lactate dehydrogenase and NADH and monitoring the absorbance change at 340 nm. Unbound ATP was determined by using luciferase–luciferin (Hackney & Clark, 1985).

ATP Chase and Quench Measurements. ATP chase experiments were done in the pH 7 buffer at 0 °C and initiated by the addition of [γ-^{32}P]ATP to a rapidly stirred solution of myosin or S1. After the indicated times, a cold chase of unlabeled ATP was added to a final concentration of 1.5 mM, and the solution was incubated on ice for 20–30 min to hydrolyze ATP bound irreversibly to myosin. The reaction was stopped with 1 M HCl containing 2 mM carrier P_i, and the remaining [γ-^{32}P]ATP was isolated from [^{32}P]P_i by molybdate extraction as described in Hackney and Clark (1985). In other cases the reaction was directly quenched with HCl without a cold chase.

Theoretical Calculations. The theoretical mass distributions for scrambling experiments and for quench experiments were calculated by numerical integration of the rate equations for the hydrolysis scheme shown in eq 1, treating each isotopic species as a distinct intermediate. The Δt values were reduced until no change in the results was noted on further reduction.

Adenylate Kinase. Adenylate kinase was assayed at 0 °C in the pH 7 buffer supplemented with 17 mM glucose, 0.3 mg/mL hexokinase and [β-^{32}P]ADP. Reactions were quenched with HCl to 1 M and heated to 100 °C for 15 min to hydrolyze ADP and ATP, and the water layer was counted for [^{32}P]glucose 6-phosphate after addition of molybdate and extraction with isobutyl alcohol/benzene (Hackney & Clark, 1985) to remove P_i. Control experiments indicated essentially complete extraction of the ^{32}P if hexokinase or S1 was deleted and complete retention of the ^{32}P if the complete reaction was supplemented with excess commercial adenylate kinase.

Results

Analysis of [β-^{17}O,γ-^{18}O]ATP and PRPP Synthetase Controls. Table I shows the distributions of phosphate samples from control experiments done for analysis of the isotopic composition of the [β-^{17}O,γ-^{18}O]ATP. Sample 1 gives the composition of the starting [^{17}O]P_i used in the synthesis of the β-position of the labeled ATP. The experimental distribution is consistent with random labeling of the P_i with 53.0% ^{17}O and 36.9% ^{18}O. Sample 2 gives the distribution of the [^{18}O]P_i used to synthesize the γ-position of the labeled ATP. From the presence of a peak at +7 mass units, it can be seen that there is a small percentage of ^{17}O in the starting [^{18}O]phosphate. The theoretical fit to the data was calculated by using 90.3% ^{18}O and 0.33% ^{17}O. Sample 3 is phosphate obtained by treatment of the labeled ATP with S1 CaATPase, which cleaves the γ-P_i with no exchange of these oxygens (Sleep & Boyer, 1978). This sample also shows a small abundance at mass +5 that is consistent with ^{17}O present in the γ-oxygens. The theoretical distribution given in the table was calculated by using 89.3% ^{18}O and 0.33% ^{17}O, which is approximately

Table II: PIX Analysis of Myosin[a]

sample	time (s)	DAPP (0.01 mM)	distribution mass above all ^{16}O species (%) 0	1	2	3	4	5	6	7	8
			Myosin, 0 °C, pH 7								
1a	4.2	-	12.2	0.38	62.5	0.07	9.61	0.03	6.19	0.11	8.88
1b	36	-	12.0	0.77	75.4	0.25	1.38	0.36	3.22	0.28	6.18
			S1, 0 °C, pH 7								
2a	6	-	4.49	0.39	33.2	0.28	12.6	0.41	17.5	0.69	30.4
2b	12	-	5.92	0.45	43.5	0.32	7.30	0.61	15.2	0.75	26.0
2c	18	-	6.8	0.44	52.5	1.08	6.19	2.22	11.5	1.47	17.9
3a	20	-	4.61	0.66	33.4	1.46	9.07	2.95	17.6	1.97	28.3
3b	20	+	5.32	0.40	38.4	0.19	7.51	0.38	16.9	0.80	30.2
4a	20	-	9.46	0.83	59.8	1.76	9.36	3.02	7.15	1.49	7.08
4b	20	+	11.8	0.93	73.1	0.17	4.01	0.17	3.95	0.20	5.66
			S1, 25 °C, pH 8								
5a	2.5	+	11.9	0.71	85.4	0.15	0.46	0.20	0.44	0.08	0.71
5b	5.0	+	12.2	0.81	85.2	0.15	0.37	0.19	0.38	0.09	0.64
6	theory, unscrambled, complete washout		10.4	0.33	89.3	0	0	0	0	0	0
7	theory, ATP from adenylate kinase		0.32	1.04	5.07	11.9	21.7	25.3	21.1	10.5	3.07
8	total P_i (sample 1b)		85.6	0.07	6.81	0.40	4.05	0.29	2.76		
	theory		87.4	0.01	7.08	0.01	3.66	0	1.86		
9	S1, 0 °C, pH 7, steady state		77.6		10.6		7.15		4.59		

[a] ATP was reacted with myosin or papain EDTA S1 as described in the text and then analyzed for PIX as indicated in Scheme I. Myosin (S1) and ATP were at 22.9 and 16.9 μM in 1a, at 30.2 and 22.7 μM in 1b, at 200 and 150 μM in series 2, at 705 and 529 μM in series 3, at 705 and 352 μM in series 4, and at 464 and 327 μM in series 5. Total reaction volumes were 70, 99.5, 18, 24, and 32.5 mL in series 1 and 2, respectively, 11 mL in series 3 and 4, and 45.5 mL in series 5. The experimental samples were corrected for natural abundance by subtracting 0.11% and 0.61% of the mass +2 peak from mass +3 and mass +4, respectively, adding these back to mass +2 and then subtracting 0.14% and 0.82% of the mass +0 peak from mass +1 and mass +2, respectively, and adding these back to mass +0.

the same composition as the starting $[^{18}O]P_i$.

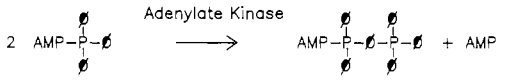
Phosphate samples 4–7 are obtained from labeled ATP that has been carried through the analysis of Scheme I and correspond to cases A, D, C, and F, respectively, of Scheme II after allowance for the less than 100% isotopic enrichments in the experimental samples. Sample 4 was obtained from untreated ATP and thus has undergone no scrambling or washout. The β,γ-bridge oxygen should be at the same average enrichment as the three γ-nonbridge oxygens because of the method of synthesis. Inclusion of this bridge oxygen with the P_i released from the PRPP should yield mainly the species at mass +8 corresponding to all four oxygens labeled with ^{18}O, and this is observed. Furthermore, there is little contribution from the odd masses, indicating that there has been no scrambling of the β-nonbridge ^{17}O into the bridge position. The calculated distribution is that for uniform labeling of all four γ-oxygens with 89.3% ^{18}O and 0.33% ^{17}O.

Sample 5 is from ATP incubated with creatine and creatine kinase, which reversibly forms creatine phosphate and free ADP, and produces complete PIX without any washout. Now the odd masses make a major contribution to the distribution due to the incorporation of ^{17}O into the β,γ-bridge position. Samples 6 and 7 were obtained by cleavage of the ATP with S1 CaATPase to generate free ADP followed by rephosphorylation to ATP with $[^{18}O]$PEP or unenriched PEP, respectively. Since free ADP is generated, which contains the original β-nonbridge and β,γ-bridge oxygens, the re-formed ATP will appear to have undergone complete PIX with either complete washout of the γ-nonbridge oxygens in sample 7 or conversion to the same distribution as the $[^{18}O]$PEP in sample 6. The observed distributions are consistent with this expectation, and the distribution of sample 7 defines the average composition of the three β-oxygens of the ADP. The theoretical distributions for samples 4–7 were all calculated with an average composition of 38.5% ^{17}O and 26.8% ^{18}O for the β-nonbridge oxygens of the starting unscrambled ATP. The internal consistency is good for these different types of controls, and this labeling pattern for the starting ATP was used in all subsequent calculations. This enrichment of the β-nonbridge position represents some loss of label from the $[^{17}O]P_i$ used in the synthesis, but no change in the $^{17}O/^{18}O$ ratio.

PIX by Myosin. Table II indicates the results of PIX experiments performed by addition of approximately stoichiometric amounts of labeled ATP to myosin or S1 followed by isolation of the residual ATP from M.ATP and analysis via Scheme I. As with the samples in Table I, β-nonbridge to β,γ-bridge scrambling is indicated by an increase in the odd masses. These PIX experiments were conducted with both intact myosin and subfragment 1 at different ATP/myosin ratios and at both 0 °C, pH 7, and 25 °C, pH 8. In all cases, washout of the γ-nonbridge oxygens is extensive for the bulk of the ATP as indicated by a major shift from the starting distribution with a maximum at +8 mass units to distributions with a maximum at +2 mass units. There is, however, no corresponding increase in the +1 mass, indicating that this washout has occurred without significant PIX and resulted in ATP with all three γ-nonbridge oxygens ^{16}O-labeled and the β,γ-bridge oxygen still labeled with the original ^{18}O. This corresponds to case E of Scheme II and, for comparison, the theoretical distribution is given in sample 6 for ATP of the <100% enrichment levels used in these incubations. This may be contrasted with the other extreme, complete washout with complete PIX, as shown in sample 7 of Table I.

The slight overabundance of the +0 mass relative to the +1 mass in some samples probably represents contamination with unenriched P_i in these small samples, and the ratio of the +1/+2 masses provides the best estimate of the extent of PIX for the ATP which has undergone washout. When a ratio of 0.004 is used for no PIX in sample 6 of Table II and a value of 0.51 for complete PIX in sample 7 of Table I, the observed data indicate only 1.2% PIX for myosin at 36 s in sample 1b; similar levels are observed with S1. Given the small size of the contribution at mass +1, it is possible that small systematic errors may bias this estimation. For example, the corrections

Scheme III



that must be applied for fragments with m/e values below 99 are small in an absolute sense but significant compared to the value of mass +1. Also, it is possible that the ^{17}O-enrichment of the bridge oxygen is slightly greater than that of the average of all four γ-oxygens in sample 4 of Table I, and no control is available that independently measures the enrichment of the bridge position. These complications can be largely eliminated by comparison of incubations for increasing times that were performed contemporaneously. Thus, the change in the +1/+2 ratio between samples 1a and 1b represents 0.8% PIX in 32 s at 0 °C, while this ratio actually drops slightly in series 2 and represents a PIX of only 0.24% in 2.5 s at 25 °C in series 5. This represents essentially the limit of detectability for the technique, with the data at 25 °C corresponding to a scrambling rate constant of 0.001 s^{-1} and a scrambling probability of 0.00007 per reversal of ATP hydrolysis using a K_2 value of 12 (Hackney & Clark, 1985) and a k_{-2} value of 15 s^{-1}. Regardless of the exact estimate of the scrambling rate, it is clearly negligible compared to the rate of resynthesis of bound ATP and significantly slower than even net product release at approximately 0.05 s^{-1}.

PIX in Un-Washed-Out ATP. The complete distributions cannot, however, be explained solely on the basis of a uniform population of myosin sites. In particular, too much unexchanged ATP remains at long reaction times, and the ATP, which has not undergone washout, has undergone significant apparent PIX in the absence, but not in the presence, of DAPP to inhibit adenylate kinase. This apparent PIX in the absence of DAPP is indicated by the elevated levels of the +3, +5, and +7 masses and is best illustrated by samples 3 and 4, which are matched sets with and without DAPP. In sample 3b with DAPP the total of the odd masses is 1.6%, which is in the range of the starting ATP (1.65%; line 4, Table I) and fully washed-out unscrambled ATP (≥0.4%; line 7, Table I, and line 6, Table II). In contrast, the total of the odd masses for sample 3a without DAPP is 6.8%, representing 20–25% total apparent PIX, which is within the range of values reported by Geeves et al. (1980). A similar pattern is observed in samples 4a and 4b, while myosin that has been purified by ion-exchange chromatography exhibits little apparent PIX even in the absence of DAPP (samples 1a and 1b). This apparent PIX, seen principally in the +3, +5, and +7 masses, is likely due to contaminant adenylate kinase activity which can convert the [β-^{17}O]ADP released from myosin into [β,γ-^{17}O]ATP (Scheme III).

This interpretation is supported by the observation with 0.025 mM free ADP at 0 °C and pH 7 of an adenylate kinase activity in S1 of 0.1 min^{-1} that is totally inhibited by 0.001 mM DAPP. It is difficult to calculate the amount of ATP expected to be synthesized by adenylate kinase during a PIX experiment due to the apparently complex kinetics of ADP release from myosin and the second-order dependence of adenylate kinase activity on the free ADP level, but this level of activity is in the range required to account for the observed PIX in the absence of DAPP.

Further indication of the role of adenylate kinase is given by detailed analysis of the mass distributions. Line 7 of Table II gives the distribution expected for ATP synthesized by adenylate kinase; there is a large contribution of the odd masses with a maximum at mass +5. The distributions with S1 in the absence of DAPP follow this pattern with mass +5 being

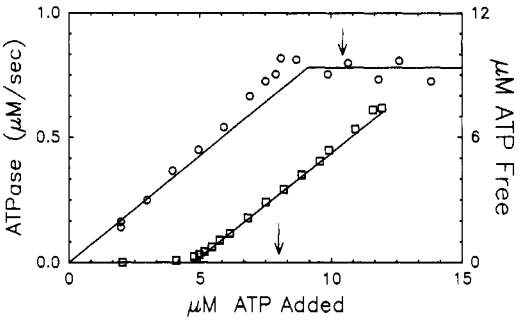


FIGURE 1: Steady-state titrations of S1. S1 was titrated with MgATP in pH 7 buffer in the presence of pyruvate kinase and PEP (Hackney & Clark, 1985). (Circles) ATPase of 10.5 μM S1 at 25 °C; (squares) free ATP with 8 μM S1 at room temperature. Arrows indicate the concentration of myosin head groups determined from the total protein concentration.

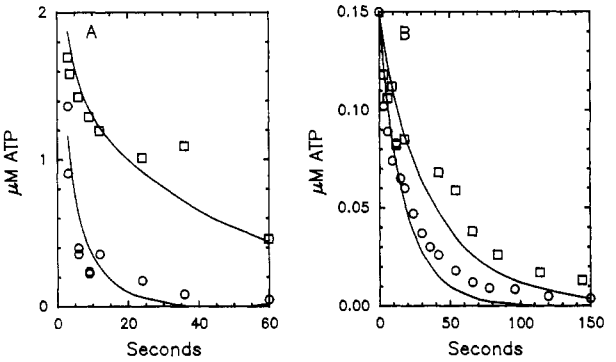


FIGURE 2: Cold chase and quench. At 0 °C in pH 7 buffer. (Circles) cold chase; (squares) HCl quench. (A) Column-purified myosin at 4 μM sites and 3.5 μM or 3.0 μM [^{32}P]ATP for cold chase and HCl quench, respectively. (B) S1 at 0.418 μM sites and ATP at 0.15 μM. The theoretical curves were calculated by numerical integration using $k_1 = 0.15 \times 10^6$ M^{-1} s^{-1} and k_{-1}, k_2, k_{-2}, k_3, k_{-3}, k_4, and k_{-4} = 0, 2.9, 2.4, 0.037, 0, 0.025, and 0 s^{-1}, respectively, for myosin. The same rate constants were used for S1 except for k_3 at 0.043 s^{-1}.

the major odd species, in contrast to case D of Scheme II for scrambling without washout, which produces mainly mass +7, and case C for limited washout, which would produce a much larger increase in the +6/+8 ratio than is observed.

Un-Washed-Out ATP. One explanation for the unwashed-out ATP is that it is merely free ATP in excess of the stoichiometry of myosin active sites. For column-purified myosin the concentration of myosin active sites is equal to the molar concentration of myosin head groups, but chymotryptic S1 has been shown to be heterogeneous and have stoichiometries of tight ATP binding sites that are slightly less than the molar concentration of myosin head groups (Hackney & Clark, 1985). Figure 1 indicates that the papain EDTA S1 used in series 2 is also heterogeneous with a nonlinear dependence of the steady-state rate on added ATP in the presence of pyruvate kinase and PEP and a stoichiometry of tight ATP binding sites of only 60% as measured by luciferase titration of free ATP. All rates for S1 are expressed in terms of the stoichiometry of these tight ATP binding sites. Thus in samples 2a–c, the apparent excess of S1 sites (200 μM) over ATP (150 μM) based on the total protein concentration actually represents an excess of ATP over tight ATP binding sites (120 μM), and most of the un-washed-out ATP is free ATP. This is also true for samples 3a and 3b.

More detailed information can be obtained from analysis of irreversible ATP binding using a cold chase of nonradioactive ATP following a pulse with radioactive ATP (Bagshaw & Trentham, 1973) as shown in Figure 2. In this experiment any radioactive ATP irreversibly bound to myosin

at the time of the cold chase will be completely hydrolyzed during the chase period, while free or loosely bound ATP will be diluted with excess cold carrier and remain largely unhydrolyzed. In the modeling, k_{-1}, k_{-3}, and k_{-4} were assumed to be zero and k_4 was determined by pyrophosphate displacement to be 0.025 s^{-1}. A value of 1.2 for K_2 was determined as the best fit to all of the HCl quench data (Figure 2 and other similar experiments not shown). For myosin, k_3 was taken as 0.037 s^{-1} and for S1 as 0.043 s^{-1} to yield the observed steady-state rates of 0.011 s^{-1} for myosin and 0.012 s^{-1} for S1. As shown in Figure 2, irreversible ATP binding is approximately described by a rate constant of 0.15×10^6 M^{-1} s^{-1}, in good agreement with the results of Chock et al. (1979) at 5 °C. These experiments were done at low myosin and ATP concentrations in order to prolong the binding phase. The values for k_2 of 2.9 s^{-1} and for k_{-2} of 2.41 s^{-1} were chosen for the best fit to the exchange data in Table II with emphasis on sample 8, which is the total P_i isolated following acid quenching in sample 1b. These values for k_2 and k_{-2} are consistent with the value of 6–9 s^{-1} reported by Taylor (1977) for $k_2 + k_{-2}$ at 3 °C. The steady-state oxygen exchange done at 0 °C using [^{18}O]PEP also places a limit on these rate constants. By use of S1 as an example, the data of sample 9 for P_i produced by steady-state hydrolysis give a value for $P_c = 0.97$, where $P_c = k_{-2}/(k_{-2} + k_3)$. With $k_3 = 0.043$ s^{-1}, this gives $k_{-2} = 1.4$ s^{-1}, which is in reasonable agreement with the value used in the calculations.

The theoretical predictions given in Figure 2 and Table II were obtained by numerical integration of eq 1 using the above set of rate constants. The fit of the observed data to the simulation is reasonable at short times in Figure 2, in agreement with the results of Webb and Trentham (1981b), but the fit is poor at longer times and a small pool of ATP that is not irreversibly bound persists beyond the time required for complete ATP binding to a uniform class of sites. This remaining ATP is not inert to hydrolysis and is completely hydrolyzed in 20 min in the absence of the cold chase. The data for ATP obtained by direct acid quenching are also included and indicate in Figure 2A that approximately 10% of the remaining ATP is not irreversibly bound by myosin at 36–60 s. This agrees with the 10% of the recovered ATP that has not undergone washout in sample 1b, and it is likely that both represent the same pool of ATP. It should be noted that although this pool of ATP represents 10% of the ATP recovered in sample 1b, this corresponds to only 2% of the initial ATP concentration.

Discussion

The sequential treatment of ATP with PRPP synthetase and base as shown in Scheme I enables the β,γ-bridge oxygen to be retained in the product P_i along with the three γ-nonbridge oxygens. This method thus results in ATP degradation with cleavage of the βP–OγP bond and directly separates the β-nonbridge and β,γ-bridge oxygens. By labeling the four γ-oxygens with ^{18}O and the β-oxygens with ^{17}O, it is possible to simultaneously observe washout of the γ-nonbridge oxygens and PIX of the β-oxygens. This method is very sensitive in that PIX is measured against an unscrambled blank which is close to zero and rises to 26% for complete scrambling of the ATP used here.[2] More importantly, however, analysis of the entire distribution from mass +0 to mass +8 for the double ^{17}O,^{18}O-labeled ATP makes it possible to observe heterogeneity that might not otherwise be detected. This is well illustrated here where a clearly bimodal distribution is observed with two populations of ATP that have undergone either complete washout in the virtual absence of PIX or, alternatively, little washout, but with significant PIX in the absence of DAPP.

The data in Table II indicate that the major pathway for reaction of myosin with ATP involves extensive washout of the γ-oxygens with little scrambling of the β-oxygens. For both myosin and S1 the washout of the γ-nonbridge oxygens is rapid, resulting in extensive accumulation of the +2 mass species even at the earliest times, while little accumulation of mass +1 (indicative of scrambling) occurs even at long incubation times. The pronounced inhibition by DAPP of the apparent PIX in the un-washed-out ATP indicates that the apparent PIX is due to contaminant adenylate kinase activity, and this activity could account for the PIX observed by Geeves et al. (1980).

The negligible rate of PIX by myosin is consistent either with the inability of eq 1 to describe the mechanism or with the bound ADP in eq 1 having essentially no tortional freedom to scramble its three β-nonbridge oxygens. One alternative mechanism (Young et al., 1974) accounts for washout of the γ-nonbridge oxygens of ATP on the basis of direct displacement by water oxygen without cleavage of the β,γ-bond. This associative mechanism is consistent with the lack of PIX by myosin but is difficult to reconcile with other known properties of phospho-transfer enzymes. Thus, myosin (Webb & Trentham, 1980) and the mitochondrial ATPase (Webb et al., 1980) hydrolyze γ-thioATP with inversion while the sarcoplasmic reticulum ATPase (Webb & Trentham, 1981a) proceeds with retention due to double displacement; the chloroplast ATPase catalyzes ATP–water exchange with significant PIX (Wimmer & Rose, 1977); the X-ray crystal structures and stereochemistry of a number of other phosphoryl transferases are consistent with simple in-line displacement of the leaving group by the nucleophile. Restricted rotation of the bound ADP, in contrast, is consistent with all of the known properties including the likelihood of coordination of the bound ADP to Mg^{2+}.

The apparent lack of torsional freedom observed here for ADP bound to myosin and also observed to a partial extent with the chloroplast ATPase (Wimmer & Rose, 1977) is in marked contrast to the high degree of torsional freedom usually characteristic of the bound P_i in similar enzyme–products complexes (Hackney, 1980; Sleep et al., 1980; Sines & Hackney, 1986). This difference is readily understandable, however, in terms of the different requirements of binding at the two sites for maximum catalysis. ATP hydrolysis occurs with net inversion, and the active site must therefore have sufficient flexibility to accommodate large changes in the geometry of the P_i subsite as it is converted from a tetracoordinate γ-phosphoryl of ATP through a pentacoordinate or metaphosphate intermediate and then back to a tetracoordinate P_i. Transition-state-analogue theory in fact postulates that the active site is optimized to bind the transition state, which is likely to have a different geometry from both the ATP and the P_i. Thus, binding of P_i and the γ-phosphoryl of ATP are not optimally strong, and the weaker nature of this binding allows for the rapid torsional movements that produce washout during reversible hydrolysis. The β-phosphoryl position, however, is not required to undergo any large geometric changes in the course of ATP formation, and its binding site geometry and metal coordination are likely optimized to fix

[2] If only the scrambling rate needs to be determined, the sensitivity could be increased even more by synthesizing ATP labeled with ^{18}O exclusively in the β-nonbridge position. This has the advantage that ^{18}O is available at higher enrichment than ^{17}O but has the disadvantage that the ability to detect washout and heterogeneity is lost.

the β-phosphoryl group strongly in the particular orientation most favorable for reaction.

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