Initial Velocity and Equilibrium Kinetics of Myokinase*

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SUMMARY
The mechanism of myokinase (ATP-AMP phosphotransferase, EC 2.7.4.3) from rabbit muscle has been investigated by studies of equilibrium isotope exchange and initial velocities. The mechanism for the myokinase reaction is random bi bi. Under most conditions, equilibrium isotope exchange between AMP and ADP is faster than that between ATP and ADP. The rate-limiting steps in the reaction include the dissociations of AMP, ADP, and ATP from the enzyme. Myokinase has two binding sites, one for magnesium-chelated nucleotides and the other for unchelated nucleotides. Michaelis constants for the four reactants are presented. AMP and ADP can form inhibitory complexes of the "dead end" type. Adenosine 5'-monosulfate is a competitive inhibitor with respect to AMP.

Studies of equilibrium kinetics of an enzyme reaction can provide evidence for the order of binding and release of reactants (1, 2). This approach has been used to elucidate the mechanisms of the hexokinase (3), alcohol dehydrogenase (4), lactate dehydrogenase (5), and glutamine synthetase (6) reactions, among others. The present paper describes the application of equilibrium kinetics to the myokinase reaction. Some initial reaction velocity studies are also reported.

Myokinase from rabbit muscle (7-10) is specific for adenine and cytidine nucleotides, and requires magnesium or manganese ions for activity. Adenosine 5'-monosulfate is a competitive inhibitor. Myokinase from bovine liver mitochondria shows similar properties (11).

EXPERIMENTAL PROCEDURE
Materials
Myokinase from rabbit muscle, with a specific activity of 200 to 750 μmoles per mg of protein per min, pyruvate kinase, hexokinase, glucose 6-phosphate dehydrogenase, and adenosine 5'-monosulfate were obtained from Calbiochem, Los Angeles. Lactate dehydrogenase, crystallized from chicken heart, was obtained from Mr. Johannes Everse of this department. Adenine nucleotides were purchased from Sigma Chemical Company, St. Louis, Missouri, and P-L Biochemicals, Milwaukee, Wisconsin. 3H-ADP in 50% ethanolic solution, with a specific activity of approximately 1.1 C per mmole, was obtained from Schwarz Bioresearch, Inc., Orangeburg, New York. 3H-AMP was obtained in the course of purifying 3H-ADP by paper chromatography. The purified 3H-ADP contained 1.5% AMP and 0.6% ATP. The 3H-AMP contained 0.5% ADP. The barium salt of AMS was purified and converted to the Tris salt by chromatography on Dowex 1 formate. The AMS so obtained gave a single spot on paper chromatography in the solvent system described below.

Methods
Paper Chromatography—Sheets of Whatman No. 3 paper (46.5 × 57 cm) were washed with 250 ml of 1 mM EDTA by descending chromatography and then dried. The solutions to be analyzed were spotted, allowed to dry, and subjected to descending chromatography with the use of 60 ml of isobutyric acid-ammonia-water solvent (12) per sheet. The paper was dried for 36 to 48 hours. The spots or strips of adenine nucleotides were located under ultraviolet light and cut out. RF values for AMP, ADP, ATP, and AMS were 0.36, 0.26, 0.20, and 0.31, respectively. AMS migrated between AMP and ADP and overlapped both.

Rate of Isotope Exchange at Equilibrium—Reaction mixtures contained 10 mM Tris-HCl buffer, 10 mM cysteine-HCl (freshly dissolved and neutralized with NaOH to about pH 8), and 0.5 mg of bovine serum albumin per ml. The final pH was 8.0 ± 0.1, unless otherwise stated. The adenine nucleotides were added as AMP in all experiments, except those shown in Figs. 2, 5, and 6, in which they were added as a mixture of AMP, ADP, and ATP. Myokinase was then added and the reaction was allowed to approach equilibrium. After at least 2 hours of incubation at 25°, about 2.5 μmoles of 3H-ADP or 3H-AMP (about 10⁶ cpm) were added per ml of reaction mixture. Unless otherwise indicated, the exchange reaction was started by ad-

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dition of 3H-ADP. Aliquots of 0.1 ml were removed at 1, 2, 3, 4, 9, 15, 60, 90, and 120 min after addition of the labeled nucleotide, except in the case of the experiment shown in Fig. 2 below in which the first seven samples were taken at 1, 2, 5, 7, 10, 15, and 30 min. Each sample was pipetted immediately into an equal volume of 10% (w/v) trichloracetic acid at room temperature. Two aliquots of 10 μl each were spotted on the chromatography paper within 1 hour after the sample was taken. Hydrolysis of ATP and ADP was found to be 2.5% and 0.3%, respectively, after incubation in 5% trichloracetic acid at 25° for 1 hour.

Myokinase was added in a concentration which was usually sufficient to cause the reaction to go at least half-way toward equilibrium in less than 15 min. The only exceptions were the experiments shown in Fig. 4, when the MgSO₄ concentration was 0.05 and 0.10 mM, and in Fig. 8, when the pH was 4.10 and 4.48. Here the reactions were so slow that they did not even reach isotope exchange equilibrium by the end of the incubation period. The equilibrium positions assumed in these four cases are indicated in the legends to Figs. 4 and 8.

After the reaction was stopped, reaction mixtures were subjected to paper chromatography as described above, and the spots containing the nucleotides were cut out and counted in a mixture of 0.75 ml of water and 20 ml of scintillation fluid contained in polyethylene vials. The scintillation fluid contained 100 g of naphthalene, 0.3 g of p-bis[2-(5-phenyloxazolyl)]benzene, and 7 g of 2,5-diphenyloxazole dissolved in dioxane and made to 1 liter with this solvent. Each sample was counted once for 4 min within 24 hours after the scintillation fluid had been added. The counting rate decreased by about 10% overnight; the reason for this decrease was not determined. Each set of AMP, ADP, and ATP spots which resulted from a single sample was counted before the next set. As a consequence the slight drift in the counting rate which occurred during the period of counting was negligible in terms of the percentage distribution of radioactivity in the three spots obtained from each sample.

The amount of radioactivity applied per spot was usually between 7,000 and 12,000 cpm; the minimum and maximum amounts were 2,000 and 20,000 cpm. In experiments with 3H-ADP, the AMP and ATP spots obtained from the shortest reaction time (1 min) contained 100 to 500 cpm.

Computation of Exchange Rates—The concentrations of reactants in each experiment were calculated from the percentage radioactivity in each spot at isotope exchange equilibrium and the total concentration of adenine nucleotide. Two methods were used to compute the isotope exchange rates, and a third method was used to check the second method. The first was a manual computation based on the assumption that, for a limited initial period of the reaction, both exchanges are independent of one another, and that each follows normal first order kinetics.

In the second method we made use of the equation derived by Wahl and Bonner (13). Applied to the myokinase reactions this takes the forms shown in Equations 1 and 2:

$$\Delta \alpha = R(\beta/D - \alpha/M)\Delta t$$

$$\Delta \gamma = K(\gamma/D - \gamma/T)\Delta t$$

where α, β, and γ are the percentage radioactivities of AMP, ADP, and ATP, respectively. (For other definitions, see Footnote 1). The reaction was simulated by iteration of Equations 1 and 2 at 1 min intervals to fit a set of computed points to the set of experimental points until the total of the differences between the two was less than 1% of the sum of the experimental points in a given curve. Only results analyzed by the second method are actually quoted in this paper. The application of this method to four of the best experiments is shown in Fig. 1. In the third method, the simultaneous integral of Equations 1 and 2 was used to compute the percentage activities of AMP and ATP at various times. The integration was kindly done for us by Dr. Max Chretien. The fitting of experimental points was done by a weighted least square method and was considered to be adequate when the corresponding R values for the computed data of two successive calculations differed by less than 0.5%. A comparison of the rates determined by the second and third methods showed the differences to be less than 5% in almost all cases.

In the rates computed by the third method, the error of the experimental points from the best fitting curve is less than 15% in five-sixths of all cases.

Measurements of Initial Reaction Velocities of Forward Reaction: 2ADP → AMP + ATP—This reaction was assayed by coupling the production of ADP to the hexokinase and glucose 6-phosphate dehydrogenase reactions. The assay mixture contained 50 mM Tris-HCl buffer, pH 8.0, 10 mM cysteine-HCl (freshly dissolved and neutralized to about pH 8), 0.5 mg of bovine serum albumin per ml, 5 mM glucose, 0.025 mM TPN, hexokinase, glucose 6-phosphate dehydrogenase, MgSO₄, and ADP. The reaction was started by the addition of 0.025 μg of myokinase per ml. The final reaction volume was 1 ml. The reaction mixtures were incubated in a water bath at 24° prior to addition of myokinase. The reaction rate was determined by measuring the increase in absorbance at 340 μm with time. Hexokinase and glucose 6-phosphate dehydrogenase were added in quantities sufficient to give a rate of TPN reduction greater than 0.32 μm per min when 1 mM ATP and 0.5 mM MgSO₄ were present in the reaction mixture. The maximum initial reaction velocity assayed was less than 4% of this rate, and the rate of TPNH formation was constant in less than 1 min after the reaction was started.

Measurements of Reverse Reaction: AMP + ATP → 2ADP—This reaction was assayed by coupling the production of ADP to the pyruvate kinase and lactate dehydrogenase reactions. The assay mixtures contained 50 mM Tris-HCl buffer, pH 8.0, 10 mM cysteine-HCl (freshly dissolved and neutralized to about pH 8), 0.5 mg of bovine serum albumin per ml, 75 mM KCl (to activate pyruvate kinase), 1 mM phosphoenolpyruvate, 10 mM MgSO₄, 0.084 mM DPNH, pyruvate kinase, lactate dehydrogenase, AMP, and ATP. The reaction was started by the addition of 0.025 μg of myokinase per ml. The reaction mixtures were equilibrated at 24° prior to addition of myokinase. The reaction rate was determined by measuring the decrease in absorbance at 340 μm with time.

Pyruvate kinase and lactate dehydrogenase were added in quantities sufficient to oxidize the added DPNH completely in 10 to 12 sec when 1 mM ADP was present in the reaction mixture. This corresponds to a rate greater than 0.3 μm per min, which is more than 12 times greater than the fastest rate assayed by this method. The reaction rates were constant during the period of observation (4 to 5 min).

RESULTS

Equilibrium Kinetics—Fig. 2 illustrates the effect of adenine nucleotide concentrations on the rates of exchange beginning...
Fig. 1. Exchange of radioactive ADP (A, B, and C) or radioactive AMP (D) with AMP (●) and ATP (■) at chemical equilibrium as a function of time. The four examples shown were chosen from 80 experiments. Reaction mixtures contained myokinase, 0.5 mg of BSA per ml, AMP, ADP, ATP, MgSO₄, 10 mM cysteine, and 10 mM Tris-HCl, pH 8.0. The temperature was 25°. Additional experimental details are given in “Experimental Procedure.” The relative concentrations of AMP, ADP, and ATP were: A, 0.739, 0.231, and 0.030; B, 0.041, 0.194, and 0.765; C, 0.321, 0.381, and 0.298; and D, 0.346, 0.362, and 0.292, respectively. The total concentrations of the nucleotides in Experiments A, B, C, and D were 4.55, 4.95, 1.00, and 2.78 mM, respectively. The concentration of MgSO₄ was the same as the total nucleotide concentration. In A, B, and C, [3H]-ADP was added at zero time; in D, [3H]-AMP was added at zero time. The first 15 or 30 min of a 2-hour isotope exchange reaction are shown. The lines were computed by iteration of Equations 1 and 2 (second method) to give the best fit to the points.

The exchange rates are proportional to the enzyme concentration. In the experiment shown in Fig. 3, they correspond to an AMP-ADP rate and an ATP-ADP rate of 0.252 and 0.157 pmole per pg of enzyme per min, respectively. Under approximately the same conditions the initial velocity of the forward reaction was 0.365 μmoles per μg of enzyme per min, which is about 1.5 times greater than the AMP-ADP equilibrium exchange rate. All other experiments were performed with myokinase preparations of about twice this specific activity.

The effect of [MgSO₄] on the exchange rates is shown in Fig. 4. The exchange rates increase to a maximum at 0.5 mM MgSO₄ and decline to a plateau at higher MgSO₄ concentrations. The maximum rate is attained at a MgSO₄ concentration which

with either [3H]-AMP or [3H]-ADP. The concentrations of AMP, ADP, and ATP at the half-maximal exchange rate are between 0.025 and 0.05 mM. The AMP-ADP exchange rate (R) is about 50% greater than the ATP-ADP exchange rate (R') over a wide range of ADP concentrations when [AMP]/[ATP] is about 1. This shows that the AMP or ADP bound to the AMP site dissociates at a faster rate than does the ATP or ADP bound to the ATP site. This result excludes an ordered reaction pathway,² for if an ordered pathway occurred one of the exchange rates would approach zero at high substrate concentrations. Random or ping pong pathways, however, are not excluded (Scheme 1).

² The terminology used is that of Cleland (14).
Fig. 2. Effect of ADP concentration on the rates of AMP-ADP and ATP-ADP isotope exchange at equilibrium. The reaction mixture contained myokinase, 0.5 mg of BSA per ml, AMP, ADP, ATP, MgSO₄, 10 mM cysteine, and 10 mM Tris-HCl, pH 8.0. The temperature was 25°C. Additional experimental details are given in “Experimental Procedure.” The total concentration of nucleotides was varied between 0.05 and 20 mM. The ratio, [AMP]:[ATP], was maintained between 1.1 and 1.3 except for the two points at 0.34 and 2.19 mM ADP, for which the ratios were 1.68 and 0.91, respectively. The concentration of MgSO₄ was equal to the total nucleotide concentration in each tube. The myokinase concentration was chosen to give a convenient rate; it ranged from 0.025 to 2.5 μg per ml. All points are adjusted to a myokinase concentration of 1 μg per ml. After equilibrium was attained the exchange reaction was started by addition of "H-AMP (○) or "H-ADP (●, □). The results show the AMP-ADP exchange (○ and □) and the ATP-ADP exchange (● and ■). The data of A which fall between 0 and 1 mm are also shown in B. In B, the solid and dashed lines connect the open and solid points, respectively.

corresponds roughly to the ATP concentration plus one-half the ADP concentration. These findings agree with those of Noda (8), who reported that the initial velocities were greatest when [Mg⁺⁺]:[ADP] and [Mg⁺⁺]:[ATP] were 0.5 and 1.0, respectively.

The equilibrium constant (Equation 3), determined from the radioactivities of AMP, ADP, and ATP at isotopic exchange

\[
K_{eq} = \frac{[AMP]}{[ATP]} \frac{[ADP]^{3}}
\]  

(3)

Fig. 3. Effect of myokinase concentration on the rates of AMP-ADP (○) and ATP-ADP (■) isotope exchange at equilibrium. The reaction mixtures contained myokinase, 0.5 mg of BSA per ml, AMP, ADP, ATP, 1 mM MgSO₄, 10 mM cysteine, and 10 mM Tris-HCl, pH 8.0. The temperature was 25°C. The total nucleotide concentration was 1 mM. The ratio, [AMP]:[ATP], was between 1.12 and 1.20. Additional experimental details appear in “Experimental Procedure.”
equilibrium, changes substantially over the range of [MgSO₄] studied. At a total adenine nucleotide concentration of 1.2 mM and at MgSO₄ concentrations of 0.2, 0.5, 1.0, and 10 mM the equilibrium constants computed from Equation 3 were 0.3, 0.8, 1.0, and 0.3, respectively. The equilibrium constants computed with the use of Equation 4 for the same points were 4.3, 3.7, 4.1, and 3.6, respectively.³

\[
K_{eq} = \frac{[\text{Mg-ATP}][\text{AMP}]}{[\text{Mg-ADP}][\text{ADP}]} \tag{4}
\]

The effect of changing [AMP]:[ATP] on the two exchange rates at a total nucleotide concentration of 0.5 mM is shown in Fig. 5. Although the ratio, [AMP]:[ATP], was varied over a 500-fold range, R was always greater than \(R'\). If the myokinase reaction involves a ping pong mechanism, the rate of the AMP-ADP exchange would increase as the ratio [AMP]:[ATP] is increased. Furthermore, the rate of ATP-ADP exchange would decrease as that ratio is increased. As both exchange rates roughly parallel each other over the whole range of [AMP]:[ATP], a ping pong mechanism can be ruled out.

The effect of changing [AMP]:[ATP] on the exchange rates at a total nucleotide concentration of 5 mM is shown in Fig. 6. The parallel decrease in exchange rates with increasing [AMP]:[ATP] is consistent with a random bi bi pathway in which AMP and ADP form inhibitor complexes of the dead end type. The change in the ratio of \(R\) to \(R'\), from 1.45 to 3.29 when the ratio of [AMP] to [ATP] is changed from 0.056 to 42 constitutes evidence for this hypothesis. Most points on this curve represent saturating nucleotide concentrations, and the change in \(R\):\(R'\) can be ascribed to a specific inhibition of the ATP-ADP exchange. As the concentration of ATP is decreased relative to the concentrations of ADP and AMP, both ADP and AMP will compete more and more effectively for the ATP site. This leads to an increase in the rate of the AMP-ADP exchange relative to the rate of ATP-ADP exchange, provided the rate-limiting step for the AMP-ADP exchange is not the covalent bond-breaking step.

At high ratios of [AMP] to [ATP] the extent of inhibition of the AMP-ADP exchange is greater when the total nucleotide concentration is 5 mM (Fig. 6) than when it is 0.5 mM (Fig. 5). The inhibition may be due to competition by Mg-AMP and Mg-ADP for the site which normally binds the unchelated nucleotide. At high ratios of [AMP] to [ATP], the ratio of [Mg-AMP] to [AMP] is 0.32 and the ratio of [Mg-ADP] to [ADP] is 8.0 in the case of Fig. 6. The corresponding ratios are 0.041 and 1.0 in the case of Fig. 5.

![Fig. 4](http://www.jbc.org/) The effect of MgSO₄ concentration on the rates of AMP-ADP (●) and ATP-ADP (■) isotope exchange at equilibrium. The reaction mixtures contained 0.10 μg of myokinase per ml; 0.5 mg of BSA per ml; AMP, ADP, and ATP totaling 1.2 mM; MgSO₄; 10 mM cysteine; and 10 mM Tris-HCl, pH 8.0. The temperature was 25°C. Chemical equilibrium was approached from ADP at MgSO₄ concentrations of 0.2, 0.5, 1.0, and 10 mM. The errors introduced by this assumption will not significantly change the shape of the curve shown. The maxima, which occur at a MgSO₄ concentration of 0.35 mM, correspond to AMP, ADP, and ATP concentrations of 0.386, 0.440, and 0.374 mM, respectively. One half of the ADP concentration plus the ATP concentration equals 0.594 mM.

![Fig. 5](http://www.jbc.org/) Effect of changing [AMP]:[ATP] at a total nucleotide concentration of 0.5 mM on the rates of AMP-ADP (●) and ATP-ADP (■) isotope exchange at equilibrium. The reaction mixture contained 0.05 μg of myokinase per ml, 0.5 mg of BSA per ml; AMP, ADP, ATP, 0.5 mM MgSO₄, 10 mM cysteine, and 10 mM Tris-HCl, pH 8.0. The temperature was 25°C. Additional experimental details are given in “Experimental Procedure.” The concentrations of AMP at [AMP]:[ATP] ratios of 0.045, 0.074, and 0.13 were 0.025, 0.038, and 0.063 mM, respectively. The concentrations of ATP at the ratios of 8.36, 11.9, and 26.1 were 0.016, 0.031, and 0.018 mM, respectively. Solid and open points show experiments done on two different days.
Fig. 6. Effect of changing [AMP]:[ATP] at 5 mM total nucleotide concentration on the rates of AMP-ADP (○) and ATP-ADP (■) isotope exchange at equilibrium. The reaction mixture contained 0.5 µg of myokinase per ml, 0.5 mg of BSA per ml, AMP, ADP, ATP, 5 mM MgSO₄, 10 mM cysteine, and 10 mM Tris-HCl, pH 8.0. The temperature was 25°C. Additional experimental details are given in "Experimental Procedure." The ratio, [AMP]:[ATP], varied from 0.066 to 42. The concentration of AMP at the [AMP]:[ATP] ratio of 0.066 was 0.200 mM. The concentration of ATP at the [AMP]:[ATP] ratio of 42 was 0.086 mM. At all other points, all three nucleotides were present in approximately saturating concentrations.

Fig. 7. Effect of adenosine 5′-monosulfate on the rates of AMP-ADP (○) and ATP-ADP (■) isotope exchange at equilibrium and on the ratio of these rates (R:R′) (▲). The reaction mixture contained 0.1 µg of myokinase per ml; 0.5 mg of BSA per ml; AMP, ADP, and ATP totaling 1 mM; 1 mM MgSO₄; AMS; 10 mM cysteine; and 10 mM Tris-HCl, pH 7.7. The temperature was 25°C. Additional experimental details are given in "Experimental Procedure." The ratio, [AMP]:[ATP], was between 0.95 and 1.15 for all points. No correction was made for ionic strength. No exchange of label from 3H-ADP into AMS was found.

Fig. 7 shows the effect of the AMP′ analogue AMS on the rate of the exchange reaction. The ratio of rates, R:R′, changes from 1.8 in the absence of AMS to 1.2 in the presence of 100 mM AMS. In addition, AMS causes a substantial inhibition of both exchange rates. There is a plateau in the initial portion of the R′ curve, but not in the corresponding portion of the R curve. These results suggest the formation of dead end complex involving the types SED₂ and SET (Scheme 2), where S represents adenosine 5′-monosulfate. The decreases in R and R′ of about 60 to 80% over this range of AMS concentration constitutes evidence for such complexes. That AMS is binding specifically to the AMP site is suggested by the substantial increase in R′ relative to R. In the absence of AMS, the rate of dissociation of

Scheme 2. Dead end inhibitor complexes for the random bi bi mechanism shown in Scheme 1.

Fig. 8. Effect of pH on the rates of AMP-ADP (○) and ATP-ADP (■) isotope exchange at equilibrium. The reaction mixture contained 0.1 or 0.2 µg of myokinase per ml; 0.5 mg of BSA per ml; AMP, ADP, and ATP totaling 1 mM; 1 mM MgSO₄; 10 mM cysteine; and 10 mM buffer. The temperature was 25°C. Additional experimental details are given in "Experimental Procedure." The buffers used were Tris-acetate from pH 4.1 to pH 5.4, Tris-maleate from 5.9 to 8.5, Tris-HCl from 7.3 to 8.5, Tris-glycine from 9.0 to 9.5, and NaOH-glycine from 9.9 to 11.0. All rates are corrected to 0.1 µg of myokinase per ml and to constant ADP concentration. For the purpose of computing exchange rates, the relative equilibrium concentrations of the adenine nucleotides for the points at pH 4.1 and 4.5 were assumed to be those of the point at pH 5.1, because the isotope exchange reactions at pH 4.1 and 4.5 did not achieve isotope equilibrium by the end of the assay period.
ATP is a function of $1/k_{\text{cat}}$ and $1/k_{-1}$ (Scheme 1). When AMS is present, the formation of SET and SED also permits dissociation of magnesium chelates of ATP and ADP to give SE (Scheme 2). In this case, the rate of dissociation is increased by the additional terms $1/k_{+1}$ and $1/k_{-1}$. The plateau in the $R'$ curve is in harmony with this hypothesis.

The effect of pH on the exchange reactions is shown in Fig. 8. There is a broad plateau between pH 7.5 and 9.5 for $R'$, and a higher but less broad plateau between pH 8 and 9 for $R$. Both curves are similar to those reported by Noda (8) for initial velocity measurements. The apparently equal exchange rates which are observed at low pH values can be attributed to low true substrate concentrations (2).

Concentrations of tripolyphosphate up to 25 mM showed little effect on the two exchange rates at equilibrium.

**Forward Reaction Kinetics**—An attempt was made to determine $K_m$ values for free ADP and Mg-ADP chelate by studying the rates of ATP production by varying the total ADP concentration at two concentrations of MgSO$_4$ (Table I). In the range of substrate concentrations used, the rate varies more rapidly as a function of the concentration of Mg-ADP than as a function of the concentration of ADP. For example, at 0.081 to 0.083 mM Mg-ADP a 25-fold increase in [ADP], from 0.017 to 0.42 mM, results in an increase in rate of only 69%. Conversely, at 0.001 mM ATP a 14-fold increase in [Mg-ADP], from 0.029 to 0.40 mM, results in a 9-fold increase in rate. Approximate $K_m$ values estimated from the data shown in Table I are 0.3 and 0.01 mM for Mg-ADP and ADP, respectively.

**Reverse Reaction Kinetics**—Fig. 9A shows reciprocal plots of reaction rates at various constant concentrations of ATP as a function of the AMP concentration. The intercepts of the straight lines with the abscissa show that the apparent $K_m$ value for AMP changes as a function of the concentration of ATP. These values correspond to 0.16, 0.14, 0.11, and 0.067 mM AMP at ATP concentrations of 0.106, 0.127, 0.270, and 1.08 mM, respectively. Similar plots with ATP as the variable at several constant concentrations of AMP are shown in Fig. 9B. Again the varying intercepts indicate that the apparent $K_m$ value for ATP varies as a function of AMP concentration. These values correspond to 0.11, 0.085, 0.067, and 0.045 mM ATP at AMP concentrations of 0.095, 0.153, 0.24, and 0.96 mM. Noda (8) and Callaghan and Weber (9) reported $K_m$ values of about 0.30 mM for both AMP and ATP. As is discussed later, the results shown in Fig. 9 are consistent with both ordered and random bi bi pathways.

AMS is a competitive inhibitor with respect to AMP (Fig. 10, A and B). The $K_i$ value for AMS is about 6.8 mM. In addition, AMS is a noncompetitive inhibitor with respect to ATP (Fig. 10, C and D).

Inhibition of the reaction by high concentrations of AMP is shown in Fig. 11. The $K_i$ for AMP is about 50 mM. If Mg-AMP is the competitive inhibitor and free AMP has little or no effect, the $K_i$ for Mg-AMP is about 5 mM. At the nucleotide concentrations used, the formation of Mg-AMP has no substantial effect on the concentration of Mg-ATP$^2$. 

![Fig. 9. Lineweaver-Burk plots of initial velocity and AMP or ATP concentration. The reaction mixtures contained 0.025 μg of myokinase per ml, pyruvate kinase, lactate dehydrogenase, 0.5 mg of BSA per ml, AMP, ATP, 10 mM MgSO$_4$, 1 mM phosphoenolpyruvate, 0.084 mM DPNH, 75 mM KCl, 10 mM cysteine, and 50 mM Tris-HCl, pH 8.0. The temperature was 24°. In A the concentrations of ATP were 0.106 (○), 0.127 (●), 0.27 (□), and 1.08 (■) mM. In B the concentrations of AMP were 0.095 (○), 0.153 (●), 0.24 (□), and 0.96 (■) mM.](http://www.jbc.org/)

<table>
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<th>Table 1</th>
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<td><strong>Total [ADP] (mM)</strong></td>
<td>[ADP$^+$]</td>
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FIG. 10. Lineweaver-Burk plots of initial velocity and AMP or ATP concentration in the presence (■) or absence (○) of 5.0 mM AMS. Experimental conditions were the same as for Fig. 9, except for the concentrations of AMP, ATP, and AMS. In A and B, the concentrations of ATP were 0.103 and 1.14 mM, respectively. In C and D, the concentrations of AMP were 0.103 and 1.00 mM, respectively.

FIG. 11. Lineweaver-Burk plot of initial velocity and AMP concentration in the range from 0.5 to 15.6 mM. Experimental conditions were the same as for Fig. 9, except for the concentrations of AMP and ATP. The concentrations of ATP were 0.25 (●) and 0.50 (□) mM.

DISCUSSION

Three general types of mechanisms, random, ordered, and ping pong, will be considered (Scheme 1). Rate laws for the random and ordered isotope exchange reaction have been derived by Boyer and Silverstein (2). Rate laws for the initial velocities of ordered and ping pong mechanisms (Equations 5a and 5b) have been provided by Cleland (14) (a, b, c, and d are constants)

\[
\frac{1}{v} = \frac{1}{a} + \frac{1}{b[M]} + \frac{1}{c[T]} + \frac{1}{d[MT]} \quad (5a)
\]

\[
\frac{1}{v} = \frac{1}{a} + \frac{1}{b[M]} + \frac{1}{c[T]} \quad (5b)
\]

The rate law for the initial rate in a random bi bi mechanism is shown in Equation 6

\[
\frac{E_t}{v} = \frac{a}{b} + \frac{c}{A_0} + \frac{d}{B_0} + \frac{e}{A_0B_0}
\]

where \(A\) and \(B\) are substrate concentrations, \(E_t\) is the total enzyme concentration, and

\[
a = \frac{k_{-1}k_{-4} + k_{-1}k_{-2} + \frac{\eta + k_{-3}}{\mu}}{k_{-2}k_{-3}/k_9} \\
b = \eta(k_{-1}k_{-4} + k_{-1}k_{-2}) + k_{-2}k_{-3} \\
c = k_{-2}k_{-3} \left( \frac{K_i}{R} + 1 \right) (k_{-2} + \mu) \\
d = k_{-2}k_{-3} \left( \frac{A}{A_0} + 1 \right) (k_{-2} + \mu) \\
e = \left[ k_{-2} + k_{-3} \right] \left( 1 + \frac{B}{K_i} + \frac{A}{K_i} \right)
\]

\[
\theta = k_{-1}k_{-2} + k_{-1}A + k_{-2}k_{-3} + k_{-4} + k_{-5}R \\
\mu = k_{-4}(k_{-6} + k_{-3}) + k_{-5}(k_{-7} + k_{-8}R) \\
\eta = k_{-6} + k_{-7} + k_{-8}
\]

\(K_i = k_{-1}/k_{-4}, \quad K_i = k_{-2}/k_{-3}, \quad \text{etc.}\)

This equation was derived with the aid of a computer program (written by D.G.R.) based on the King-Altman method (19). It should be noted that Equation 6 contains substrate concentration terms in the numerator and denominator. The presence
of these terms is due to a multicyclic reaction pathway (Scheme 1) in contrast to the uniclyclic pathways of the ordered and ping pong mechanisms.

The results in Fig. 2 show that the rates of both exchange reactions remain at constant ratios over a wide range of substrate concentrations. In such cases, an ordered mechanism can be eliminated (2, 3). A reaction mechanism of the ping pong type (Equation 5b) yields a series of parallel lines when the reciprocal of the initial velocity is plotted against the reciprocal of the second substrate (14). Furthermore, in a ping pong mechanism the rates of the two exchange reactions pass through maxima at very high and very low [AMP]:[ATP] ratios. Neither requirement for a ping pong mechanism is met, and a reaction pathway of the random type must therefore be operating. The initial velocity data and the isotope exchange data at equilibrium are consistent with the rate laws derived for a random mechanism.

The results of the kinetic measurements at equilibrium can be used to deduce the relative magnitudes of the dissociation constants of the random mechanism shown in Scheme 1. Equations 3 and 4 of Boyer and Silverstein (2) may be rewritten in the form of Equations 7 and 8.

$$\frac{R}{E_i} = \frac{k_a}{k_a + k_2 (k_2 + k,T)} \left( \frac{k_2}{k_2 + k_1} \right)$$

$$\times \left[ \frac{1}{k_2} \left( 1 + \frac{k_2}{D_3} + \frac{K_a K_2}{D_2 D_3} \right) + \frac{1}{k_2} \left( 1 + \frac{K_a}{T} + \frac{K_2}{M} \right) \right]^{-1}$$

$$\frac{R'}{E_i} = \frac{k_a}{k_a + k_4 (k_4 + k,M)}$$

$$\times \left[ \frac{1}{k_4} \left( 1 + \frac{k_4}{D_3} + \frac{K_a K_2}{D_2 D_3} \right) + \frac{1}{k_4} \left( 1 + \frac{K_a}{T} + \frac{K_2}{M} \right) \right]^{-1}$$

The concentration-dependent terms and the letters which later will be substituted for them are

$$a = k_2 / (k_1 + k,M)$$

$$b = k_2 / (k_a + k,T)$$

$$c = k_2 / (k_a + k,M)$$

$$d = k_2 / (k_1 + k,M)$$

These terms have values between 0 and 1, depending on whether the corresponding reactant concentration is high or low, respectively. The contribution of the second major term of Equations 7 and 8 may be cancelled out by dividing $R$ by $R'$. Substitution of $a$, $b$, $c$, and $d$ yields Equation 9.

$$\frac{R}{R'} = \frac{k_2}{k_2 + 1 + \frac{K_a}{T} + \frac{K_2}{M}}$$

It is possible to approximate the relative magnitudes of the various rate constants with the use of the data in Table II. Since the two exchange rates, $R$ and $R'$, differ, the phosphate transfer step is not rate limiting with respect to both exchanges. Two general cases remain to be considered.

In the first of these cases, the covalent bond-breaking steps ($k_9$ and $k_{-9}$) are rate limiting with respect to the AMP-ADP exchange ($k_{-4}$ and $k_4$) only. This case implies that

$$k_{-4} > k_4$$

$$k_{-9} > k_9$$

Under these conditions the denominator of Equation 9 approaches 1. Equation 9 then becomes

$$\frac{R}{R'} = \frac{k_9}{k_9 + 1 + \frac{K_a}{T} + \frac{K_2}{M}}$$

This corresponds to the case for which the ratio $R:R' = 1$. (Line i in Table II). In the case of Lines ii and iii (Table II), Equation 10 can be further simplified by eliminating the $a$ term, because $[AMP^2]$ is high relative to the $K_m$ value.

$$\frac{R}{R'} = \frac{k_9}{k_9 + 1 + \frac{K_a}{T} + \frac{K_2}{M}}$$

The results in Table II do not fit Equations 10 and 11 under the specified conditions. For example, substituting 0.090 and 0.047 for $[ADP]^2$ in the $c$ term of Equation 11 leads to a larger $R:R'$ ratio for ii than for iii, in contrast to what is found experimentally. It follows that the covalent bond-breaking steps are not rate limiting for either exchange.

In the second case, the dissociation steps for the AMP-ADP and the ATP-ADP exchanges are rate limiting. This case implies that

$$k_{-4} \text{ and/or } k_4 \ll k_9$$

$$k_{-9} \text{ and/or } k_9 \ll k_9$$

The terms containing reactant concentrations which are large relative to the $K_m$ value can be deleted. Under these conditions Equation 9 simplifies to Equations 12 to 14 for the three ratios of $R$ to $R'$ given in Table II. These equations correspond to

<table>
<thead>
<tr>
<th>Ratio of reactants for three points in Fig. 6</th>
<th>Ratio of reactants for three points in Fig. 6</th>
<th>Concentration</th>
</tr>
</thead>
</table>
| $.\begin{array}{llll}
   \text{Ratio} & \text{Ratio} & \text{Concentration} \\
   \text{[AMP]} & \text{to [ATP]} & \text{R to R'} & \text{[AMP]} & \text{[ATP]} & \text{[ADP]} & \text{[AMP]} & \text{[ATP]} & \text{[ADP]} & \text{[AMP]} & \text{[ATP]} & \text{[ADP]} \\
   \text{1} & 0.045 & 1.1 & 0.024 & 0.024 & 0.024 & 0.133 & 0.133 & 0.133 & 0.024 & 0.024 & 0.024 \\
   \text{ii} & 1.1 & 1.5 & 0.162 & 0.137 & 0.137 & 0.090 & 0.090 & 0.090 & 0.065 & 0.065 & 0.065 \\
   \text{iii} & 27.0 & 3.5 & 0.451 & 0.017 & 0.017 & 0.047 & 0.047 & 0.047 & 0.046 & 0.046 & 0.046 \\
\end{array}$ |
and are consistent with the data for low, intermediate, and high ratios of [AMP] to [ATP].

\[
\frac{R}{R'} = 1.1 = \frac{k_{-8} + k_{-4}}{k_{-3} + k_{-8} + ck_{-4}} + 1 + \frac{k_0}{k_{-3} + k_{-8} + ck_{-4}} \quad (12)
\]

\[
\frac{R}{R'} = 1.5 = \frac{k_{-9} + k_{-2} + k_{-4}}{k_{-9} + k_{-1} + k_{-2} + ck_{-4}} + 1 + \frac{k_0}{k_{-9} + k_{-1} + k_{-2} + ck_{-4}} \quad (13)
\]

\[
\frac{R}{R'} = 3.5 = \frac{k_{-9} + 1 + k_3 + c + d}{k_{-9} + k_{-8} + ck_{-4}} + 1 + \frac{k_0}{k_{-9} + k_{-8} + ck_{-4}} \quad (14)
\]

Assuming an arbitrary value of 10 sec\(^{-1}\) for the rate constants \(k_0\) and \(k_{-8}\), a solution for these equations yields \(k_{-8} = 8\), \(k_{-4} = 0.3\), \(k_{-2} = 0.4\), and \(k_{-1} = 1.55\) sec\(^{-1}\). Other combinations of solutions can be found which also fit. All solutions presented obey the following conditions. In Equation 14, the ratio, \(k_{-2}\):\(k_{-8}\), must be more than 3 times greater than \(k_{-1}\):\(k_{-2}\), assuming the terms containing \(c\) and \(d\) approximately equal 1. If \(d\) is much less than 1, then the magnitude of \(k_{-8}\) must be increased substantially relative to \(k_{-2}\). In Equation 13 the second fraction in the denominator must be several times the corresponding term in the numerator. For this condition to exist, \(k_{-4}\) must be small with respect to \(k_{-2}\) and \(d\) must be small. When this is true, the term \(ck_{-4}\) makes only a very small contribution. Then \(k_{-4}\) is small relative to \(k_{-2}\) and \(d\) is small relative to \(k_{-2}\). These conditions require the phosphate transfer step to be substantially faster than the rate-limiting dissociation step.

Previous workers provided evidence that the two substrates in each direction of the myokinase reaction are a chelated and an unchelated nucleotide, and that the chelated and unchelated substrates have their own binding sites. The enzyme operates at maximum rate in one direction when at least half of the ADP is chelated, or in the other direction when all of the ATP present is chelated (8). The data presented in Fig. 4 are consistent with these findings. Our results favor a random bi bii mechanism, which requires two binding sites per catalytic site.

The substrate inhibition by AMP (Fig. 11) suggests the existence of an AMP \(E^*\) AMP complex. The only way in which AMP can inhibit the reaction is by binding at the ATP site. If the ATP site binds only magnesium-nucleotide chelate, then the inhibitory form of AMP must be Mg-AMP. Support for this interpretation is found in the work of Noda (8) who showed that the rate of the reverse reaction falls off substantially when the concentration of Mg\(^{2+}\) rises above that of ATP. Under these conditions, Mg-AMP can be formed in significant concentrations.

Fig. 6 also provides evidence for inhibition of the reaction by excess AMP, since \(R\) and \(R'\) decrease together with increasing [AMP]:[ATP]. The curves predicted by Equations 7 and 8 under conditions of high reactant concentrations are parallel lines with constant exchange rates. This contrasts with the decrease in exchange rates with increasing [AMP]:[ATP] observed experimentally (Fig. 6). A possible explanation is that AMP and ADP are forming inhibitory complexes. When AMP is occupying the nonchelated binding site, the main inhibitory complex in the experiment illustrated in Fig. 6 may be ADP-E-AMP. This hypothesis is supported by the observation that in the case of initial velocity measurements a 30% inhibition of the rate of ADP production occurs in the presence of 15 mM AMP (4.9 mM Mg-AMP), and 0.25 mM ATP (Fig. 11), whereas under equilibrium conditions a 50% decrease in the isotope exchange rate, \(R\), occurs with 3.8 mM AMP (0.77 mM Mg-AMP), 1.55 mM ADP (1.34 mM Mg-ADP) and 0.34 mM ATP (Fig. 6). The 30% inhibition is probably due to formation of the enzyme complex AMP-E-AMP, and the 50% inhibition can be attributed to the complex ADP-E-AMP.

Fig. 7 shows that AMS inhibits primarily the AMP-ADP exchange. If AMS were to bind to both sites in significant quantities then the specificity of the AMS inhibition with respect to AMP would probably not be observed. Both \(R\) and \(R'\) would be expected to decrease sharply on addition of AMS. Only \(R\) decreases sharply, indicating a substantial specificity of the binding of AMS. This strongly supports the hypothesis of two binding sites per catalytic site on the enzyme.

Many of the foregoing conclusions are based on the equilibrium isotope exchange studies. They would be difficult if not impossible to draw from initial velocity data.

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