31P NMR Quantitation of the Displacement of Equilibria of Arginine, Creatine, Pyruvate, and 3-P-Glycerate Kinase Reactions by Substitution of Sulfur for Oxygen in the β Phosphate of ATP*

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31P NMR measurements have been found to be a convenient means for simultaneously measuring the concentrations of several species in the equilibrium mixtures of the reactions catalyzed by arginine kinase and creatine kinase.

\[ \text{MgATP} + X \rightleftharpoons \text{MgADP} + XP \]

where \( X \) = arginine or creatine and \( XP = P\text{-}
arginine or P-creatine. The free energy of phosphorylation of various metabolites by adenosine 5'-O-(2-thiotriphosphate) at pH 8.0 and 30°C is more exergonic than the corresponding phosphorylations by ATP by about 2.5 kcal/mol, resulting in a displacement of the equilibrium toward the nucleoside diphosphates by a factor of approximately 60. Since this factor does not depend on the nature of the metabolite, the equilibrium constants of thionucleotide reactions may be used to determine the equilibrium constants of corresponding oxynucleotide reactions which lie too far toward ATP. The equilibrium constants of the oxynucleotide reactions catalyzed by pyruvate kinase and 3-P-glycerate kinase calculated by this method from the experimentally determined equilibrium constants of the corresponding thionucleotide reactions are 3.1 \times 10^{-4} and 2.9 \times 10^{-4}, respectively, under the experimental conditions used. The equilibrium constants and degree of stereoselectivity of the arginine kinase reaction are altered when \( Ca^{2+} \) replaces \( Mg^{2+} \) as the activating metal ion.

The kinases are a class of enzymes that catalyze transfer of a phosphoryl group from a nucleoside triphosphate to a metabolite (designated \( X \) below). For adenine nucleotides, the reaction is schematically:

\[ \text{MgATP} + X \rightleftharpoons \text{MgADP} + XP \]  

(1)

The normal metabolic functions of kinases fall into three categories, with corresponding variation in the value of the equilibrium constant for Reaction 1: (a) phosphorylation of metabolites (example: hexokinase), for which \( K_{\text{ox}} \) for Reaction 1 is large, (b) ATP synthesis (examples: pyruvate kinase, 3-P-glycerate kinase), for which \( K_{\text{ox}} \) is small; and (c) regulation of concentrations of phosphorylated compounds (examples: creatine kinase, adenylate kinase), for which \( K_{\text{ox}} \) is near unity.

The free energy contributions to such a phosphoryl transfer can be separated formally into two partial reactions, \( \Delta G_1 \) for ATP hydrolysis and \( \Delta G_2 \) for XP hydrolysis:

\[ \text{MgATP} + H_2O \rightleftharpoons \text{MgADP} + P; \quad \Delta G_1 \]

(2)

\[ X + P \rightleftharpoons XP + H_2O; \quad -\Delta G_2 \]

(3)

When \( XP \) is a very high energy phosphate, the overall equilibrium constant for Reaction 1 is difficult to measure because the equilibrium concentrations of MgADP and XP are very small. It has recently been observed, however, that the equilibrium of the 3-P-glycerate kinase reaction with ATPβS in place of ATP is displaced toward the formation of ADPβS and 1,3-bis-P-glycerate (1). Since the metabolite partial reaction here is the same as when the oxynucleotides are used, the free energy difference must be due to a decrease of \( \Delta G_i \) by the substitution of sulfur. The magnitude of the alteration does not depend on the nature of the metabolite, so that the equilibrium of the thio analog reaction:

\[ \text{MgATPβS} + X \rightleftharpoons \text{MgADPβS} + XP \]

(4)

will be displaced to the right from that of the corresponding oxynucleotide reaction (Reaction 1) by the same amount for any \( X \). If we define the equilibrium constants:

\[ K_{\text{ox}} = \frac{[\text{MgADP}] [XP]}{[\text{MgATP}] [X]} \]

(5)

\[ K_{\text{thio}} = \frac{[\text{MgADPβS}] [XP]}{[\text{MgATPβS}] [X]} \]

(6)

then the ratio \( K_{\text{thio}}/K_{\text{ox}} \) will be a constant irrespective of the nature of \( X \). In cases where \( K_{\text{ox}} \) is too small to measure, \( K_{\text{thio}} \) might be large enough to give readily measurable equilibrium concentrations, and \( K_{\text{ox}} \) could then be calculated, using a value for the \( K_{\text{thio}}/K_{\text{ox}} \) ratio determined from reactions where both \( K_{\text{ox}} \) and \( K_{\text{thio}} \) are measurable.

31P NMR is an unusually direct method of measuring these concentrations, for one can observe the compounds in the equilibrium mixture itself, without any chemical perturbation or separation which might alter the position of equilibrium. We have used 31P NMR to measure \( K_{\text{ox}} \) and \( K_{\text{thio}} \) for the reactions of arginine kinase and creatine kinase, thus establishing a value of the ratio \( K_{\text{thio}}/K_{\text{ox}} \). We have extended the measurements to pyruvate kinase and 3-P-glycerate kinase,

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The abbreviations used are: ATPβS, adenosine 5'-O-(2-thiotriphosphate); ADPβS, adenosine 5'-O-(2-thiodiphosphate); ATPγS, adenosine 5'-O-(3-thiotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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where $K_{\text{thio}}$ can be measured by NMR, and $K_{\text{oxy}}$, which is too small to measure easily, is calculated.

**EXPERIMENTAL PROCEDURES**

**Materials—**D$_2$O was purchased from Aldrich and was purified by vacuum sublimation before use. Arginine, P-arginine (sodium salt), creatine, P-creatine (disodium salt), P-enolpyruvate (trimonocyclo-hexylammonium salt), acetyl phosphate (lithium potassium salt), and Hepes were from Sigma, 3-P-glycerate (trisodium salt) was obtained from Boehringer. Commercial ADP$_3$S (trilithium salt, Boehringer) contained ADP and as much as 15% AMP. It was purified by chromatography on DEAE-Sephadex A-25, eluting with a gradient from 0.1 to 0.5 M triethylammonium bicarbonate, pH 7.8, then freed from metals as described below.

ATP$_3$S was usually used as the mixture of diastereomers produced by pyruvate kinase from ADP$_3$S and P-enolpyruvate (2, 3). For some experiments with arginine kinase, pure $R$ isomer was synthesized in the reaction of ADP$_3$S with acetyl phosphate catalyzed by acetate kinase (4).

Solutions of ATP and ATP$_3$S were made metal-free by adjustment of the pH to 8 to 9 with KOH, followed by extraction with 1% 8-hydroxyquinoline in chloroform and back extraction of the aqueous layer with pure chloroform. In the case of ADP and ADP$_3$S, chloroform extraction has been found to remove significant amounts of nucleotide, so metal removal was accomplished instead by passage through Chelex 100 (Bio-Rad) at pH 8 to 9. Metal-free stock solutions of potassium salts of nucleotides were stored frozen.

Acetate kinase (Escherichia coli) was purchased from Sigma.

Lobster muscle arginine kinase was prepared as previously reported (5, 6), as was yeast 3-P-glycerate kinase (7). Creatine kinase and pyruvate kinase were both from rabbit muscle and were gifts from Dr. George Reed and Mr. David Ash.

**Methods—**pH measurements are direct meter readings with a Radiometer 26 pH meter; no correction was made for the 20% D$_2$O in solutions for NMR.

Solutions of adenine nucleotides were assayed by absorbance at 250 nm, using $e = 15.4$ (mm$^{-1}$cm$^{-1}$). Creatine, P-enolpyruvate, and 3-P-glycerate were assayed by published enzymatic methods (8). Arginine was assayed analogously to creatine, using arginine kinase in place of creatine kinase. Solutions of P-arginine and P-creatine were assayed for acid-labile phosphate by hydrolysis for 10 min at 100°C in 0.5 M sulfuric acid, followed by assay for inorganic phosphate (9), and for contamination by unphosphorylated arginine or creatine by the enzymatic methods above.

Equilibrium mixtures were usually constituted by adding enzyme to a solution of ADP (or ADP$_3$S) and phosphorylated metabolite. For 3-P-glycerate kinase, this was not convenient due to the instability of 3-glycerate-P, so the equilibrium was established starting with ATP (or ATP$_3$S) and 3-P-glycerate instead. An initial mixture of ATP$_3$S plus metabolite was used in a few other experiments as well, as described below.

A typical solution for recording $^{31}$P NMR spectra initially contained the following in a total volume of 1.0 to 1.5 ml in a 10-mm diameter NMR tube: 220 mM Hepes, 30 mM nucleotide, 30 mM metabolite, 38 mM magnesium acetate, 5 mM EDTA, 20% D$_2$O (for field/frequency lock), and 0.5 to 3.0 mg/ml of enzyme. The pH (direct meter reading) was adjusted to 8.0 with KOH in all experiments, thus making total [K$^+$]=250 mM, sufficient to activate pyruvate kinase. Some experiments with lower substrate concentrations (down to 5 mM) were also done and gave essentially the same results, but had larger errors due to lower signal-to-noise ratios.

$^{31}$P NMR spectra were obtained at 145.7 MHz in Fourier transform mode on a Bruker WH-360 spectrometer with the sample temperature controlled at 30°C. Spectral parameters were: memory size, 16,000 (8,000 real spectral data points); spectral width, 5,000 to 8,000 Hz; 100 transients; flip angle, 45°; pulse width 18 µs; acquisition time, 1.0 to 1.6 s; pulse delay, 10 s. Chemical shifts are expressed in parts per million upfield from external 85% phosphoric acid. Typical spectra appear in Fig. 1. Preliminary experiments showed that a 10-s delay was sufficient to allow relaxation to the extent that integral ratios of various resonances did not change upon further delay. It was also found that application of sensitivity enhancement of the free induction decay to the extent of 1 Hz line broadening substantially improved the signal-to-noise ratio without altering relative integrations, and this enhancement was used routinely. For each sample, spectra were repeatedly accumulated until the integrals no longer changed.

Inverse gated proton decoupling (decoupler on during acquisition, off during delay) was used to sharpen the resonances of the α phosphorus atoms of the nucleotides (coupled to H-5 of the ribose) without any Overhauser enhancement. In several cases, the α resonances were sufficiently resolved by this procedure to allow them to be integrated separately; without decoupling such resolution could not be achieved (see Fig. 2). The lack of enhancement was confirmed empirically. Under these conditions, it typically required about 18 min to obtain a spectrum.

Equilibrium constants were calculated from the relative integrals of $^{31}$P resonances at equilibrium and the initial concentrations deter-

**Fig. 1.** $^{31}$P NMR spectra (145.7 MHz) of the equilibrium mixtures of the arginine kinase reaction with oxy- and thionucleotides at pH 8.0 and 30°C in the presence of Mg$^{2+}$. Each spectrum is the result of 100 transients with a 10-s delay and inverse gated proton decoupling. A, oxynucleotide reaction initiated with 29.5 mM ADP, 29.4 mM P-arginine, 37.4 mM Mg$^{2+}$, and 4.1 mM EDTA; B, thionucleotide reaction initiated with 23.8 mM ATP$_3$S-R, 11.9 mM arginine, 11.9 mM P-arginine, 29.1 mM Mg$^{2+}$, and 3 mM EDTA.

**Fig. 2.** Effect of inverse gated proton decoupling on the NMR signal of the α-P of CaATPBS. (80% S; 20% R)
mined by standard chemical, enzymatic, or spectroscopic assays. The concentration of the metabolite \( X \) was determined by difference between the initial sum \( ([X] + [XP]) \) and the equilibrium value of \([XP] \). In the 3-P-glycerate kinase reaction, \( X \) = 3-P-glycerate, and its concentration can be determined directly from its phosphorus resonance integral, it agreed with the value determined by the difference.

Values of integrals were only reproducible to ±10% at the signal-to-noise levels obtained under these conditions. However, the integrals of the various resonances did not change independently from one spectrum to another, so that the estimated errors in the calculated equilibrium constants (reported in Table I) were usually smaller than the statistical expectation for the composite of four independent concentration measurements, each with 10% error. Errors in \( K_{Kthi} \) are greater than those in \( K_{Kxy} \) due to lower signal-to-noise ratios caused by broader resonances and to greater overlap of resonances.

**RESULTS AND DISCUSSION**

The values of equilibrium constants reported in Table I are apparent constants \( K_{Kxy} \) or \( K_{Kthi} \), as defined above. For arginine, creatine, and pyruvate kinases, these are not equal to the thermodynamic equilibrium constants because the hydrogen ion released in the reaction has been neglected. The true equilibrium constant for these three kinases is:

\[
K_{Kxy} = \frac{[\text{MgADP}][\text{XP}][\text{H}^+]}{[\text{MgATP}][\text{X}][\text{H}^+]} = K_{Kxy}^{\text{thermo}}
\]

An analogous equation applies to \( K_{Kthi} \). The apparent constants \( K_{Kxy} \) and \( K_{Kthi} \) are thus sharply pH-dependent for these three kinases. In the 3-P-glycerate kinase reaction, however, with the phosphorylation of an acidic carboxyl group, the equilibrium constant is pH-dependent only in a particular pH range; the change in hydrogen ion concentration in the reaction depends on the difference in the degree of ionization of the carboxyl and carboxyphosphate groups at the pH of the measurement.

**Arginine Kinase**—The ratio of \( K_{Kthi} \) to \( K_{Kxy} \) is 65, corresponding to a change in free energy of 2.5 kcal/mol in favor of MgADPβS. Determinations of \( K_{Kxy} \) for arginine kinase by chemical and enzymatic methods have given values of 0.31 (10) and 0.46 (11) at pH 8.0 and 30°C. These constants are difficult to compare directly to the ones obtained in the present study because of large differences in ionic strength, magnesium and substrate concentrations, and solvent (H\(_2\)O versus 20% D\(_2\)O).

The striking reversal of the position of equilibrium can be seen in the NMR spectra of Fig. 1. The detail-obscuring breadth of the resonance of the \( \beta-P \) of ATPβS appeared in all solutions where Mg\(^{2+}\) was the activating ion and is probably due to chemical exchange among various metal complexes. Addition of EDTA in excess of the magnesium causes the resonances to sharpen dramatically (Fig. 3). Further evidence that chemical exchange among complexes is responsible for the broad \( \beta-P \) resonance is the sharpening of the peak if Ca\(^{2+}\) is substituted for Mg\(^{2+}\) (Fig. 4). Since the rates of formation and dissociation of Ca\(^{2+}\) complexes are three orders of magnitude greater than Mg\(^{2+}\) (12), effective dynamic averaging of the chemical shifts is achieved by the substitution.

When the reaction was initiated with ATPβS-R and arginine, equilibration of the two diastereomers of MgATPβS in the reaction mixture is clearly observed (Fig. 1B). Isomeric equilibration occurs more slowly than the overall equilibration of the phosphoryl transfer because of the enzyme's low rate of utilization of the S isomer.

The isomeric equilibration and other features in the spectrum are even more clear when the reaction is activated by Ca\(^{2+}\) instead of Mg\(^{2+}\). The chemical shifts in the calcium complexes are such that the ATPβS diastereomers are better resolved in the \( \alpha \) and \( \gamma \) resonances than in either the magnesium or the uncomplexed nucleotides. For example, the \( \gamma-P \) resonances of ATPβS-R and -S are barely resolved in the absence of metal ion (Fig. 3). Magnesium imposes a difference in chemical shift between the \( \gamma-P \)-s of the two diastereomers almost equal to the \( \beta-P \) coupling constant, so that the lines overlap for the magnesium complex (Fig. 1B), but the shift difference for the calcium complex is only about half of \( J_{\gamma\beta} \), thus giving four well-defined lines (Fig. 4). For the \( \alpha-P \)-resonances of the diastereomers, unresolved in the absence of metal, either magnesium or calcium gives resolution of the diastereomers, but the calcium complex also shows an improved separation of the \( \alpha-P \) resonance of ATPβS from that of ADPβS compare Fig. 1B and Fig. 4). In addition, the broadening of the ADPβS \( \beta-P \) resonance does not appear with calcium due to more rapid exchange among complexes than in the case of magnesium, leading to a fast exchange narrowed signal as mentioned above.

Fig. 4 shows the time course of equilibration in a calcium-activated arginine kinase reaction mixture. The ratio of ADPβS to the sum of ATPβS diastereomers reaches an equilibrium value rapidly. Complete equilibration of the \( R \) and \( S \) isomers follows. It was observed qualitatively that the stereoselectivity of the arginine kinase reaction at the \( \beta-P \) of ATPβS was reduced when Ca\(^{2+}\) rather than Mg\(^{2+}\) was the activating ion. With magnesium, the \( S \) diastereomer reacts only very slowly, and equilibration starting with MgATPβS is difficult to achieve in reasonable times unless a large pro-

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C. L. Lerman, N. Shih, and M. Cohn, unpublished results.

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**Table I**

| \( X \) | \( K_{Kxy} \) | \( K_{Kthi} \) | \( K_{Kthi}/K_{Kxy} \) |
|--------|-------------|-------------|------------------|
| Arginine | 0.17 ± 0.02 | 11 ± 2 | 65 |
| Creatine | 0.053 ± 0.002 | 3.1 ± 1 | 58 |
| Pyruvate | 3.1 × 10\(^{-4}\) | 0.019 ± 0.004 | |
| 3-P-glycerate | 2.9 × 10\(^{-3}\) | 0.018 ± 0.003 | |

\( ^* \) Calculated using the average value of \( K_{Kthi}/K_{Kxy} = 62. \)
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...portion of the R isomer is present as in Fig. 1. With calcium, however, the S isomer is used at a rate more competitive with the R isomer. Experiments to quantitate the metal ion dependence of the rates of the arginine kinase reactions are in progress. The lesser discrimination between oxy and thio ligands by calcium is consistent with previous observations of reduced kinetic stereoselectivity in the hexokinase reaction with calcium as the activating metal ion. Values obtained were K_{oxy},...}

...-	ext{P}(ATP\beta\text{S})$ relative to MgATP\beta\text{S} (13).

The equilibrium constants of the arginine kinase reaction were also altered when calcium was used as the activating metal ion. Values obtained were $K_{oxy} = 0.22 \pm 0.02$ and $K_{thio} = 6.6 \pm 0.4$, giving a calculated free energy change of only 2.1 kcal/mol for sulfur substitution in the calcium nucleotides. This result suggests that the distribution of chelated species of calcium nucleotides and their thio analogs in solution differs from the distribution of magnesium-chelated species.

Creatine Kinase—The ratio of $K_{thio}$ to $K_{oxy}$ is 58, in good agreement with the value obtained with arginine kinase. The average of the two values is 62, and is used in calculations below and in Table I. Rather extensive investigations and discussion of the creatine kinase equilibrium constant appear in the literature (14–16) emphasizing the dependence of $K_{oxy}$ on the concentration of free magnesium ion. None of the previous studies at pH 8 was done at concentrations as high as those used in these NMR experiments (free Mg$^{2+}$ is about 3 mM). A value of $K_{oxy} = 4.5 \times 10^{-2}$ at pH 7 and 37°C can be interpolated at [Mg$^{2+}$]_{free} = 3 mM in Fig. 1 of Ref. 16, then corrected to $K_{oxy} = 4.5 \times 10^{-2}$ (at pH 8), which compares favorably with the value obtained in the current investigation by NMR.

Pyruvate Kinase—The constant $K_{thio}$ determined from $^{31}$P NMR was $1.9 \times 10^{-2}$. Applying the $K_{thio}/K_{oxy}$ ratio of 62 determined from the two guanidino kinases gives a calculated value of $K_{oxy} = 3.1 \times 10^{-4}$. A value of $4.5 \times 10^{-10}$ has been previously reported for pH 8, 30°C (17), but the reaction mixtures were constituted with 2- to 3-fold excess of nucleotides over Mg$^{2+}$. An approximate value of $3 \times 10^{-4}$ was estimated from previous NMR experiments on the oxynucleotide reaction (18).

3-P-Glycerate Kinase—Investigation of this reaction presented difficulties because of the hydrolytic lability of 1,3-bis-P-glycerate. Reactions were therefore always initiated with ATP\beta\text{S} and 3-P-glycerate. Substantial decomposition of the enzymatically produced 1,3-bis-P-glycerate to 3-P-glycerate and P, could be seen in successive spectra. It was possible to raise the enzyme concentration high enough (>1 mg/ml) that the apparent $K_{thio}$ stayed constant despite this decomposition, indicating that the equilibration of the enzymatic reaction was much faster than the hydrolysis of 1,3-bis-P-glycerate under these conditions. If the enzyme concentration was raised to 3 mg/ml, a constant value of $K_{thio}$ could be reached in less than 30 min, after which time there is still enough 1,3-bis-P-glycerate present to measure the integral of its P-1 resonance fairly accurately. The value of $K_{thio} = 1.8 \times 10^{-4}$ in Table I is derived from such an experiment.

Dividing $K_{thio}$ by 62 gives a calculated $K_{oxy} = 2.9 \times 10^{-4}$. Comparison with previously determined values is again confounded by lack of data for high free magnesium concentrations, but extrapolation of some recent data at pH 7 and 37°C (19) gives an estimated value of $2 \times 10^{-4}$.

The experimentally determined values for $K_{oxy}$ for all four kinase reactions are listed in Table I together with the observed values for $K_{oxy}$ for arginine and creatine kinases and the calculated values for pyruvate and 3-P-glycerate kinases. The finding that the free energy change in phosphoryl transfer is greater when a polyphosphate with an internal thiophosphoryl group (ATP\beta\text{S}) forms a phosphate with a terminal thiophosphate group (ADP\beta\text{S}) than in the same reaction of an ordinary polyphosphate is qualitatively consistent with an earlier observation that in the equilibrium between ATP\beta\text{S} and ATP\gamma\text{S}, the latter is greatly favored (20).

It is clear that the use of $^{31}$P NMR and the thionucleotide analogs is a rapid and convenient method for the determination of equilibrium constants of nucleotide reactions which favor the formation of nucleoside triphosphates. The method is capable of being extended to more physiological conditions (especially lower concentrations) at the expense of NMR accumulation time. It is also likely that sulfur substitution in the alpha phosphate instead of the beta of ATP in reactions which lead to formation of adenosine 5'-phosphoroethioate, such as reactions catalyzed by adenylyl transfer enzymes, e.g. acetoy-CoA and aminoaoyl-tRNA synthetases, or pyrophosphoryl transferring enzymes, e.g. P-ribosylphosphate synthetase, will displace the equilibria toward adenosine 5'-phosphoroethioate. The ability to impose metabolites with thionucleotides at steady state concentrations quite different from the normal ones with oxynucleotides may prove to be a useful tool in studies of metabolic regulation.

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