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Secondary ^{18}O Isotope Effects for Hexokinase-Catalyzed Phosphoryl Transfer from ATP†

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ABSTRACT: Secondary ^{18}O isotope effects in the γ-position of ATP have been measured on phosphoryl transfer catalyzed by yeast hexokinase in an effort to deduce the structure of the transition state. The isotope effects were measured by the remote-label method with the exocyclic amino group of adenine as the remote label. With glucose as substrate, the secondary ^{18}O isotope effect per ^{18}O was 0.9987 at pH 8.2 and 0.9965 at pH 5.3, which is below the pK of 6.15 seen in the V/K profile for MgATP. With the slow substrate 1,5-anhydro-D-glucitol, the value was 0.9976 at pH 8.2. While part of the inverse nature of the isotope effect may result from an isotope effect on binding, the more inverse values when catalysis is made more rate limiting by decreasing the pH or switching to a slower substrate suggest a dissociative transition state for phosphoryl transfer, in agreement with predictions from model chemistry. The ^{18}O equilibrium isotope effect for deprotonation of $HATP^{3-}$ is 1.0156, while Mg^{2+} coordination to ATP^{4-} does not appear to be accompanied by an ^{18}O isotope effect larger than 1.001.

Yeast hexokinase catalyzes phosphoryl transfer from MgATP to the 6-OH group of a number of furanose- and pyranose-type compounds (Viola et al., 1982). The addition of substrates is random, but with a strong preference for sugar binding first (Rudolph & Fromm, 1971; Danenburg & Cleland, 1975), and the chemical mechanism involves general base catalysis by an aspartate group which removes a proton from the 6-OH group of the substrate (Viola & Cleland, 1978; Anderson et al., 1978). The isotope partitioning studies of Rose et al. (1974) showed that in the forward direction glucose dissociates from the enzyme at a much slower rate than it undergoes reaction but that MgATP can dissociate at a relatively fast rate compared with the forward reaction.

The chemical mechanism of phosphoryl transfer from phosphate monoesters has been extensively studied over the years because of its fundamental importance in biological processes (Westheimer, 1981; Benkovic & Schray, 1978). One of the most extensively investigated questions is whether the reaction occurs with transfer of free metaphosphate. Stereochemical experiments have suggested that in certain nonnucleophilic environments, free metaphosphate can exist (Cullis & Nicholls, 1987; Freeman et al., 1987; Friedman et al., 1988). However, the use of linear free energy relationships has shown that the rates of phosphoryl transfers from various phosphoramidates and phosphate esters have an electronic dependence on the nucleophile, indicating a certain amount of interaction between the nucleophile and the transferring phosphoryl group (Skoog & Jencks, 1984; Bourne & Williams, 1984; Jencks et al., 1986). Whether the reaction occurs with the formation of metaphosphate or through an exploded transition state in an S_N2 reaction, it appears that phosphoryl transfer from monoesters of phosphate occurs via a dissociative pathway in solution.[1] Phosphate diesters, on the other hand, appear to react by mechanisms with more associative transition states as evidenced by the relatively high entropy of activation and β_{nuc} values for these compounds relative to those observed for phosphate monoesters (Benkovic & Schray, 1978).

The chemical mechanisms of phosphoryl transfer in enzyme-catalyzed reactions are still unknown. The nonpolar environment of many enzyme active sites would be conducive to a dissociative mechanism, while the close proximity of the nucleophile to the phosphoryl group in the active site and the ability of groups in the active site to stabilize the negative charges on the oxygens of the phosphoryl group would favor an associative type of mechanism. Phosphorylated thiols, which are highly reactive and should be good substrates for a dissociative reaction, have been shown to be good substrates for alkaline phosphatase, but are very poor substrates for kinases (Knight & Cleland, 1984; Knight et al., 1984). This suggests that alkaline phosphatase might catalyze phosphoryl transfer in a dissociative fashion, while the slow reaction of the thiophosphates with kinases might reflect steric constraints on a associative reaction. That alkaline phosphatase catalysis of phosphoryl transfer reactions is a dissociative process is supported by the recent finding that the secondary ^{18}O effects for this reaction are slightly inverse (Weiss & Cleland, 1989). We now report the secondary isotope effects for the hexokinase-catalyzed phosphoryl transfer from remote-labeled ATP in an attempt to determine the chemical nature of this phosphoryl transfer reaction.

Materials and Methods

Chemicals and Enzymes. Yeast hexokinase, glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides*, Tris buffer, and NAD were from Boehringer. All other enzymes were from Sigma. Triethylamine, adenosine, ATP, and 6-chloropurine riboside were from Aldrich. MES buffer was from Research Organics. $H_2{}^{18}O$ (approximately 96 atom %

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[1] Strictly speaking, a dissociative mechanism has a metaphosphate or substituted metaphosphate intermediate, while an associative mechanism has a pentacovalent phosphorane intermediate. Phosphoryl transfers which occur in a concerted S_N2 fashion will have their transition states described as dissociative when the sum of bond orders from phosphorus to entering and leaving groups is less than unity and associative when the sum of bond orders along the reaction coordinate is greater than unity. A "dissociative type" of mechanism includes one with a metaphosphate intermediate as well as concerted ones with low axial bond order in the transition state.

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^{18}O) was from Monsanto. $^{15}NH_3$ was from Merck, and $^{14}NH_4Cl$ was from Prochem. 1,5-Anhydro-D-glucitol was prepared by the method of Ness et al. (1950). Adenosine deaminase and alkaline phosphatase were dialyzed against 10 mM Tris, pH 8.2, prior to use. Phosphorus oxychloride from Aldrich was distilled from sodium under argon before use. Triethylamine and trioctylamine from Aldrich were distilled from calcium hydride under argon before use. [$^{18}O_3$]-Phosphoenolpyruvate was prepared by acid-catalyzed exchange with $H_2{}^{18}O$ (O'Neal et al., 1983).

Synthesis of Labeled Compounds. (*A*) [6-^{15}N]*Adenosine.* $^{15}NH_3$ was condensed in the break seal vial it was received in using liquid nitrogen. The seal was broken, and 180 mL of chilled anhydrous ethanol was added such that the resultant solution was approximately 10% ammonia (the solubility of ammonia in ethanol is 20% at 0 °C). The ethanolic ammonia solution was transferred to a 200-mL steel hydrogenation bomb containing 6-chloropurine riboside (10 mmol) such that the ammonia was in approximately a 3-fold excess. The bomb was sealed and placed in a heated (110 °C) shaker. After 12 h, the ethanol was removed in vacuo and the solid redissolved in water. Formic acid (88%) was added to lower the pH to 2.5–3, thus protonating the adenosine and leaving the 6-chloropurine riboside neutral. This solution was applied to a 1.5 × 25 cm column of Dowex 50 resin in the ammonium ion form. The 6-chloropurine riboside was eluted with water and the adenosine with 20 mM ammonia. Fractions were monitored by thin-layer chromatography using cellulose F microcrystalline precoated plates of 0.1-mm thickness with visualization under UV light. The fractions containing the adenosine were pooled, and the ammonia was removed in vacuo. The ^{15}N content was determined to be 99% by quantitatively deaminating a small portion of the adenosine with adenosine deaminase and analyzing the ammonia as described below.

(*B*) [γ-$^{18}O_3$,6-^{15}N]*ATP.* [6-^{15}N]Adenosine (1 mmol) was dissolved in 5 mL of triethyl phosphate by heating over an open flame. Octylamine (0.4 mL) was added to the hot solution and the solution cooled in an ice bath. Phosphorus oxychloride (0.11 mL) was added to the cold solution. After 1 h, tetrakis(tri-*N*-butylammonium) pyrophosphate (1.1 mmol dissolved in 4 mL of triethyl phosphate) was added and the solution allowed to reach room temperature. After 45 min, 4 mL of triethylamine was added and the solid isolated by centrifugation. The solid was dissolved in water and applied to a 5 × 40 cm DEAE A-25 column equilibrated in 0.1 M triethylammonium bicarbonate (TEAB), pH 7.6. The column was eluted with a 2-L 0.1–0.8 M gradient of TEAB. The [6-^{15}N]ATP (yield, 0.203 mmol) was dissolved in 0.1 M HEPES buffer, pH 7.0, and 0.5 mmol of glucose was added along with 125 units of yeast hexokinase. After 2 h, the solution was filtered by using an Amicon SP-30 filter to remove protein and the ADP isolated with a DEAE A-25 column eluted with TEAB buffer. [$^{18}O_3$]Phosphoenolpyruvate (60 mg) was added to the [6-^{15}N]ADP dissolved in 0.1 M HEPES at pH 7.0, and 100 units of pyruvate kinase was added. After 12 h, the reaction was applied to a 5 × 40 cm DEAE A-25 column and the [γ-$^{18}O_3$,6-^{15}N]ATP isolated by elution with a TEAB gradient. The yield was 146 μmol. The ^{18}O incorporation was determined by ^{31}P NMR to be 71% [γ-$^{18}O_3$]ATP and 25% [γ-$^{18}O_2$]ATP with the remainder being the monolabeled compound (89% labeling).

(*C*) [6-^{14}N]*Adenosine.* Because the most economical way to obtain $^{14}NH_3$ is from $^{14}NH_4Cl$, a method for producing dry ammonia had to be devised as any water in the system will lead to the production of inosine from the 6-chloropurine riboside. To accomplish this, a solution of potassium *tert*-butoxide in dimethyl sulfoxide was slowly added to a heated slurry of $^{14}NH_4Cl$ and dimethyl sulfoxide. The $^{14}NH_3$ that evolved was passed through glass tubing, to the end of which was attached a gas dispersion tube that was immersed in ethanol. The flask containing the ethanol was cooled in a salt–ice bath. When the solution was approximately 10% in ammonia, the ethanolic ammonia (380 mL) was placed in a 400-mL steel hydrogenation bomb containing 6-chloropurine riboside (21 mmol). In order to avoid possible contamination, the bombs, as was the case with all other glassware and columns, were not the same as those used in [6-^{15}N]adenosine synthesis. The remaining steps in the synthesis and purification of [6-^{14}N]adenosine are as described above for [6-^{15}N]-adenosine. Because [6-^{14}N]adenosine and [6-^{15}N]adenosine, as ATP, are ultimately mixed together in approximately natural abundance of ^{15}N, or 0.37%, some 100 times as much [6-^{14}N]adenosine was needed. For this reason, the synthesis of [6-^{14}N]adenosine was carried out a number of times.

(*D*) [6-^{14}N]*ATP.* This material was prepared in approximately 30% yield by using the same procedure as that for the synthesis of [6-^{15}N]ATP described previously, except that [6-^{14}N]adenosine was used rather than [6-^{15}N]adenosine. Again, since some 100 times more of this light-labeled ATP was required, a larger scale synthesis was used, and this was repeated 3 times.

(*E*) *Remote-Labeled ATP.* The heavy-labeled ATP ([γ-$^{18}O_3$,6-^{15}N]ATP) was mixed with the light-labeled ATP ([6-^{14}N]ATP) to give approximately 80% ^{15}N natural abundance at N-6. The ^{15}N content was precisely determined by using the degradation and analysis method described below. More heavy-labeled material was thus added to the calibrated 80% mixture to give remote-labeled ATP that had 0.371% ^{15}N at the exocyclic position of the adenine ring.

Determination of the ^{18}O Equilibrium Isotope Effect for Deprotonation of the γ-Phosphate of ATP. The pK of a mixture of [γ-$^{18}O_3$]ATP and unlabeled ATP was determined by plotting the chemical shift of the γ-^{31}P NMR signal of ATP versus pH (Knight et al., 1986). The fitted pK was used to determine the fractional protonation of the ^{16}O compound (X). The observed differences in the chemical shifts of the ^{16}O and the ^{18}O compounds (Y) at a given pH were then fitted to eq 1 to determine the value of $^{18}K_{eq}$ (Ellison & Robinson, 1983).

$$Y = Y_H - (\Delta\delta)X + {}^{18}K_{eq}X(\Delta\delta + Y_L - Y_H)/({}^{18}K_{eq}X + 1 - X) \quad (1)$$

In eq 1, Y_L and Y_H are the Y values for fully protonated and fully deprotonated ATP, respectively, and $\Delta\delta$ is the difference in chemical shift caused by protonation.

Determination of the ^{18}O Equilibrium Isotope Effect for Mg^{2+} Coordination of Inorganic Phosphate. In order to correct the observed ^{18}O secondary kinetic isotope effects determined for the hexokinase-catalyzed reaction, attempts were made to determine the equilibrium isotope effect for Mg^{2+} coordination to the γ-phosphate of ATP by a similar method to that described above for the deprotonation reaction. However, because of the slow dissociation of the MgATP complex on the NMR time scale, the signals became too broad. Similar results were obtained when AMP was used in place of ATP. However, it was apparent from these experiments that any isotope effect on Mg^{2+} binding to either nucleotide was small. To overcome these line-broadening problems, inorganic phosphate was used with the idea that any equilibrium ^{18}O isotope effect for the coordination of Mg^{2+} to phosphate

would serve as a good estimate for any effect on the γ-phosphate of ATP. To determine this isotope effect, $MgSO_4$ was titrated into solutions of either K_2HPO_4 and $K_2HP^{18}O_4$ in 100 mM TAPS buffer, pH 8.5, or KH_2PO_4 and $KH_2P^{18}O_4$ in 100 mM MES buffer, pH 6.4. After each addition of $MgSO_4$, both the chemical shift and the line width of each species were determined. Proton decoupling was used for all samples in an effort to obtain narrower line widths. The low-pH titration was between 0 and 1 M $MgSO_4$, while the high-pH titration was only between 0 and 30 mM due to precipitation at higher Mg^{2+} concentrations. At low pH, most of the phosphate was converted to the Mg^{2+} complex, while at high pH 26% was complexed (the range of chemical shifts for complete complex formation was −0.98 ppm at high pH and +0.69 ppm at low pH). No *significant* differences between the chemical shifts of the ^{16}O and ^{18}O species were observed at low or high pH, indicating that there is little or no ^{18}O equilibrium isotope effect on Mg^{2+} coordination to inorganic phosphate. It was estimated that any isotope effect greater than 1.0015 would have led to significant differences in the chemical shifts being observed.

Kinetic Assays. Kinetic assays to determine the relative rate of yeast hexokinase at pH 8 and pH 5 and with 1,5-anhydro-D-glucitol were performed in 3.0-mL total volume in 1-cm path-length cuvettes by measuring the absorbance at 340 nm with a Beckman DU spectrophotometer. The rates were measured by the use of coupled assays. With glucose as the substrate, glucose 6-phosphate formation was detected with glucose-6-phosphate dehydrogenase. ADP formation was detected with a pyruvate kinase/lactate dehydrogenase couple when 1,5-anhydro-D-glucitol was the substrate (Viola & Cleland, 1978; Viola et al., 1982). The relative rates determined by these methods were in agreement with those of Danenburg and Cleland (1975) and Viola et al. (1982).

Incubation of Remote-Labeled ATP and ATP with Hexokinase. The appropriate ATP was reacted at 25 °C with limiting glucose in the presence of hexokinase to 50% reaction. The reaction mixture contained ATP (80 μmol), $MgSO_4$ (100 μmol), NAD (60 μmol), glucose (40 μmol), and 120 μmol of Tris (pH 8.2) or 120 μmol of MES (pH 5.5), hexokinase (14 units), and glucose-6-phosphate dehydrogenase (120 units) in a final volume of 10 mL. The reactions with MES were then adjusted to pH 5.3 with KOH. The pH of both the MES- and Tris-buffered reactions decreased slightly during the course of the reaction. The reactions were allowed to continue at room temperature for 18 h and then frozen in liquid nitrogen and stored at −20 °C until separation of the ATP and ADP by FPLC. The reactions with 1,5-anhydro-D-glucitol were performed as described above in Tris buffer; however, no glucose-6-phosphate dehydrogenase was added, and 1,5-anhydro-D-glucitol, added in portions, was substituted for glucose. At time zero, 30 μmol was added with an additional 10 μmol added every hour for the next 5 h. The reaction was monitored by FPLC and stopped after 8 h by immersion in liquid nitrogen. The reaction was thawed and the enzyme removed by Amicon filtration (YM-10 filter) immediately prior to injection on the FPLC. When 1,5-anhyro-D-glucitol was added all at once, no reaction occurred, probably indicating a strong dead-end complex between ADP and 1,5-anhydro-D-glucitol, since high levels of 1,5-anhydro-D-glucitol could be added if the ADP was removed by coupling with pyruvate kinase. Fractional reaction was determined by the limiting reagent glucose, or in the case of the 1,5-anhydro-D-glucitol reactions, which were terminated as stated above, by end-point analysis of an aliquot of ATP and ADP using hexokinase, glucose 6-phosphate, or pyruvate kinase/lactate dehydrogenase couples, respectively.

Isolation of ATP and ADP by FPLC. The completed hexokinase reactions were diluted to 19 mL, and 9.5 mL was injected onto a Pharmacia Mono Q 10/10 column with the use of a 10-mL Superloop. The column was eluted isocratically with water at a rate of 4 mL/min for 15 min and then with a gradient to 75 mM KCl/2.5 mM Tris, pH 8.2, at 60 min. The column was eluted isocratically for 12 min and the gradient resumed to a final concentration of 210 mM KCl/7 mM Tris at 120 min. The column was then washed with 300 mM KCl/10 mM Tris for 5 min and reequilibrated with distilled water for 5 min. The approximate retention time for ADP was 75 min. ATP had a retention time of 105 min. The fractions containing ADP and those containing ATP were separately evaporated to dryness.

Isolation of N_2 from ATP and ADP. The separated ADP or ATP was dissolved in 25 mL of water, and alkaline phosphatase (≈1500 units) and adenosine deaminase (≈500 units) were added. Ammonia production was monitored spectrophotometrically at 425 nm after reaction of a small aliquot with Sigma ammonia color reagent (a modified Nessler's reaction). The reactions generally ceased to produce ammonia after 30 min, but were allowed to incubate at room temperature for 18–24 h before the ammonia was removed by alkaline steam distillation into 10 mL of 0.1 N sulfuric acid until 40 mL of distillate was collected. The distillate was evaporated to dryness and 1 mL of distilled water added. No difference either in the amount of ammonia or in the isotopic composition was observed if the enzymes were removed by using Amicon filtration prior to steam distillation. The overall yield of ammonia relative to the initial amount of ATP was 94% ± 8%. The isolated ammonia was oxidized by hypobromite oxidation to give N_2 as described by Nuiry et al. (1984) and by Hermes et al. (1985). The N_2 gas was condensed onto 5-Å molecular sieves prior to isotope ratio mass spectrometry. The mass ratios of the N_2 were determined by using a Finnigan Delta E isotope ratio mass spectrometer and are reported in Table I.

Data Analysis. Equation 2 was used to calculate the observed $^{18}(V/K)$ [actually $^{15,18,18,18}(V/K)$] isotope effects from

$$^{18}(V/K)_{obs} = [\log (1 - f)]/\{\log (1 - F) - \log [1 - f + f(R_p/R_s)]\} \quad (2)$$

the isotopic ratio of $^{15}N/^{14}N$ in products, R_p, and the isotopic ratio in substrate, R_s, after fractional reaction f. Equation 3

$$^{18}(V/K)_{obs} = [\log (1 - f)]/\{\log [1 - f(R_p/R_0)]\} \quad (3)$$

was used to calculate the observed $^{18}(V/K)$ isotope effects from the isotopic ratio of products after fractional reaction, f, and the isotopic ratio of the initial substrate before reaction (or that of the final product after 100% reaction of the substrate), R_0. Equation 4 was used to calculate the observed $^{18}(V/K)$

$$^{18}(V/K)_{obs} = [\log (1 - f)]/\{\log [(1 - f)(R_s/R_0)]\} \quad (4)$$

isotope effects from the isotopic ratio of substrate after fractional reaction f and the isotopic ratio of the initial substrate before reaction (or that of the final product after 100% reaction of the substrate), R_0. The ratios R_p, R_s, and R_0 which are shown in Table I are $^{15}N/^{14}N$ isotope ratios relative to the same tank standard of N_2, rather than isotope ratios.

The correction for incomplete incorporation of both ^{18}O and ^{15}N into the substrate was accomplished by using eq 5 where

$$^{18}(V/K) = \{(T^{1/i} - 1)/[1 - T^{1/i}(1 - y)/i]\} + 1 \quad (5)$$

$T = (P/R)/[1 - Q(P/R - 1)]$. This is a general form of the more specific equations described by Hermes et al. (1984) and

Table I: Observed Isotope Effects for Phosphoryl Transfer Catalyzed by Hexokinase

substrate	pH	f	R_s[f]	R_p[f]	observed isotope effect[c]
glucose/RL ATP[a]	8.2	0.50	1.0005	1.0077	0.9949
		0.50	1.0024	1.0042	0.9987
		0.50	0.9994	1.0081	0.9938
					mean = 0.996 ± 0.003
glucose/RL ATP	8.2	0.50	1.0180	1.0215	0.9975
		0.50	1.0147	1.0166	0.9987
		0.50	1.0155	1.0226	0.9950
					mean = 0.997 ± 0.002
glucose/RL ATP	5.3	0.50	1.0040	1.0056	0.9988
		0.50	0.9998	1.0062	0.9954
		0.50	0.9983	1.0064	0.9942
					mean = 0.996 ± 0.002
1,5-anhydro-D-glucitol/RL ATP	8.2	0.50	0.9986	1.0059	0.9945
		0.34	0.9996	1.0078	0.9934
		0.35	1.0002	1.0084	0.9934
					mean = 0.9938 ± 0.0007
glucose/RL ATP	8.2	0.0[d]	1.0020		
		1.00[e]		1.0019	
		1.00[e]		1.0024	
glucose/NA ATP[b]	8.2	0.50	1.0057	1.0051	1.0004
		0.50	1.0060	1.0054	1.0004
		0.0[d]	1.0060		
		1.00[e]		1.0055	
					mean = 1.0004
glucose/NA ATP	5.3	0.50	1.0055	1.0061	0.9995
		0.50	1.0060	1.0060	1.0000
		0.0[d]	1.0056		
		1.00[e]		1.0056	
					mean = 0.9997 ± 0.0003

[a] These observed isotope effects [$^{15,18,18,18}(V/K)$] were determined by using remote-labeled ATP (RL ATP). [b] These observed isotope effects [$^{15}(V/K)$] were determined by using natural-abundance ATP (NA ATP) and were used in eq 5 to correct the $^{15,18,18,18}(V/K)$ values. [c] These isotope effects were calculated by eq 2. [d] Substrate at 0% reaction. [e] Product of 100% reaction. These values, combined with the 0% reaction value, comprise the R_o value that, together with the R_p and R_s values shown, was used in eq 3 and 4 to calculate the $^{15,18,18,18}(V/K)$ values. The $^{15,18,18,18}(V/K)$ values are summarized in Table II. [f] These ratios, R_p, R_s, and R_o, represent the ^{15}N content of the samples relative to the same tank standard of N_2, and thus do not represent the actual isotopic content of the sample.

Weiss and Cleland (1989). In eq 5, P = the observed isotope effect with remote-labeled material calculated from eq 2, 3, or 4 [$^{15,18,18,18}(V/K)$ in this case], R = the isotope effect in remote-label position with natural-abundance material and also calculated by using eq 2, 3, or 4 [$^{15}(V/K)$ in this case], i = the number of discriminating atoms (=3 in this case), Q = $(1 - b)z/bx \approx z/b$ = the degree to which light material in the remote-labeled mixture is depleted below natural abundance, b = the fraction of double-labeled material in the remote-labeled mixture [approximately natural abundance, equal to the fraction of [γ-$^{18}O_3$,6-^{15}N]ATP in the final mixture (in this case =0.00371)], z = the fraction of heavy label present in the remote-label position of light material used for the remote-labeled mixture = the fraction of ^{15}N in [6-^{14}N]ATP (in this case =0.0001), x = the fraction of heavy label in the remote-label position of double-labeled material used for mixing to give the approximately natural abundance = the fraction of ^{15}N in [γ-$^{18}O_3$,6-^{15}N]ATP (in this case =0.995), and y = the fraction of heavy discrimating label in double-labeled material, and in the case of three discriminating atoms, this is the fraction containing all atoms labeled = the fraction of $^{18}O_3$ in [γ-$^{18}O_3$,6-^{15}N]ATP (in this case =0.71).

Table II: $^{18}(V/K)$ Isotope Effects Corrected for Binding Effects, Incomplete Incorporation, and Isotope Effects on Deprotonation

sugar	pH	$^{15,18,18,18}(V/K)$[a]	$^{18}(V/K)$[b]	$^{18}(V/K)$[c]
glucose	8.2	0.996 ± 0.002[d]		
		0.996 ± 0.004[e]		
		0.997 ± 0.002[f]		
		mean = 0.997 ± 0.004	0.9987	0.9987
glucose	5.3	0.996 ± 0.002[d]		
		0.9957 ± 0.0006[e]		
		0.997 ± 0.004[f]		
		mean = 0.996 ± 0.002	0.9986	0.9965
1,5-anhydro-D-glucitol	8.2	0.9938 ± 0.0007[d]		
		0.993 ± 0.001[e]		
		0.9948 ± 0.0007[f]		
		mean = 0.9941 ± 0.0004	0.9976	0.9976

[a] Uncorrected observed isotope effects calculated from data shown in Table I. [b] Isotope effect for a single ^{18}O per phosphate group corrected by using eq 5. [c] Isotope effect after correction for the proportion of protonated ATP, as described in the text, using eq 6. [d] Isotope effect calculated by eq 2. [e] Isotope effect calculated by using eq 3. [f] Isotope effect calculated by using eq 4.

When the isotope effects were determined at pH 8.2, nearly all of the ATP was $MgATP^{2-}$, and no correction for the presence of $HATP^{3-}$ was needed. At pH 5.3, however, a portion of the ATP will be $HATP^{3-}$, and it can be shown that the true ^{18}O isotope effect is given by

$$\text{true } ^{18}(V/K) = \frac{[\text{app } ^{18}(V/K) \text{ from eq 5}][1 + (K_{Mg}/M)(H/K)]}{1 + (K_{Mg}/M)(^{18}K_{eq}H/K)} \quad (6)$$

where M is the free Mg^{2+} concentration (3.2 mM), K_{Mg} is the dissociation constant of MgATP (25 μM at the ionic strength used; Adolphsen & Moudrianakis, 1978), H is the H^+ concentration ($10^{-5.3}$), and K is the third ionization constant of ATP ($10^{-6.63}$). $^{18}K_{eq}$ is 0.0156 times 0.89 (the degree of ^{18}O substitution in the ATP) plus 1.0. The apparent $^{18}(V/K)$ values at pH 5.3 must thus be divided by 1.00213, and this correction has been applied to the values in Table II. No correction for an ^{18}O equilibrium isotope effect on Mg^{2+}, coordination to the γ-phosphate of ATP was made, since the failure to find such an effect with phosphate suggests that it would be absent for ATP as well.

Results

^{18}O Equilibrium Isotope Effects on Protonation and Mg^{2+} Complexation of ATP. While in our experiments at pH 8.2 ATP exists almost entirely as $MgATP^{2-}$, at pH 5.3 an appreciable amount of $HATP^{3-}$ is present at the levels of free Mg^{2+} that were used, and the observed isotope effect on the hexokinase reaction must be corrected for the equilibrium ^{18}O isotope effect for protonation of ATP^{4-} and the coordination of ATP by Mg^{2+}. The value for the protonation is readily determined by titrating a mixture of [γ-$^{18}O_3$]ATP and unlabeled ATP with acid and observing the ^{31}P chemical shifts of the γ-phosphate during the conversion of ATP^{4-} to $HATP^{3-}$ (Ellison & Robinson, 1983). The titration curve is shown in Figure 1A.

The ^{18}O label causes a small upfield shift in this signal, and because of the isotope effect on the ionization constant, the peak for the labeled γ-phosphate begins to move upfield first, with the result that the two peaks move further apart and then come back together as the titration is completed (Figure 1B). A fit of these data to eq 1 gave an isotope effect on deprotonation of $HATP^{3-}$ of 1.0156 ± 0.0007 for three ^{18}O's in the γ-position, and the pK from Figure 1A was 6.66 ± 0.06. The pK agrees with that determined by Pecoraro et al. (1984 and

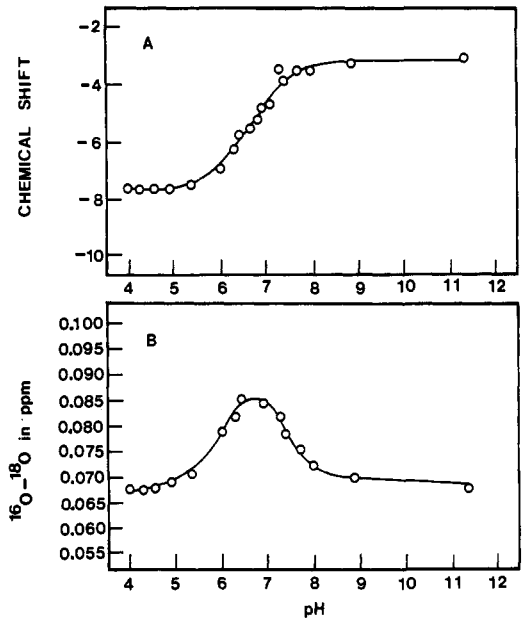

FIGURE 1: (A) Titration curve of the ^{31}P NMR chemical shift of the γ-phosphate of ATP. (B) Difference in chemical shifts versus pH for the γ-phosphate of ATP and [γ-$^{18}O_3$]ATP.

references cited therein), and the ^{18}O equilibrium isotope effect is similar to that found previously for other phosphate esters by Knight et al. (1986).

Attempts to determine the ^{18}O isotope effect caused by Mg^{2+} coordination to ATP by a similar method failed because $MgATP^{2-}$ and ATP^{4-} have nearly identical chemical shifts, and in the titration in which $MgATP^{2-}$ is converted to $HATP^{3-}$, the relatively slow dissociation of MgATP [7000 s^{-1}; see Pecoraro et al. (1984) and references cited therein] causes the ^{31}P NMR peaks of ATP to be broadened because this rate falls in the intermediate-exchange region. In an attempt to overcome these difficulties, similar experiments were carried out with AMP which forms only a monodentate complex with Mg^{2+} (dissociation constant, 10 mM) that dissociates much more rapidly; however, similar line broadening was observed. The isotope effect was thus determined by using inorganic phosphate which also forms a Mg^{2+} complex with a dissociation constant of approximately 13 mM (stability constant, 75 M^{-1}). No significant differences between the chemical shifts of the ^{16}O and ^{18}O species were observed under conditions where 26% to nearly all of the Mg^{2+} complex was formed, and thus the value of the ^{18}O equilibrium isotope effect for the Mg^{2+} coordination to inorganic phosphate was considered to be unity. Inorganic phosphate should be a reasonable model for the isotope effect of Mg^{2+} coordination to the γ-phosphate of ATP. We thus assume that there is no isotope effect on this process and that the only correction that needs to be applied to the observed secondary ^{18}O isotope effects is that for the deprotonation of $HATP^{3-}$.

The small value of the equilibrium isotope effect for Mg^{2+} coordination to ATP is supported by the work of Takeuchi et al. (1988), who determined changes in the infrared and Raman frequencies of the phosphate groups of ATP and ADP upon coordination by Mg^{2+}. They found that frequencies corresponding to asymmetric and degenerate stretching modes of the terminal phosphates increased 8–10 cm^{-1} when Mg^{2+} was coordinated while the symmetric stretching vibrational frequency increased 4–5 cm^{-1}. By contrast, protonation caused a 90 cm^{-1} increase in the symmetrical stretching frequency of the γ-phosphate of ATP. These data suggest that the equilibrium ^{18}O isotope effect of Mg^{2+} coordination should be approximately 1.001, in agreement with our experimental result.

Secondary ^{18}O Kinetic Isotope Effects on the Hexokinase Reaction. $^{18}(V/K)$ kinetic isotope effects were measured by using the remote-label technique first described by O'Leary and Marlier (1979). The remote label was ^{15}N placed in the exocyclic nitrogen of the adenine ring to monitor the isotope effect of ^{18}O substitution in the nonbridge positions of the γ-phosphate of ATP. The remote-labeled [γ-$^{18}O_3$,6-^{15}N]ATP was then mixed with ^{15}N-depleted [6-^{14}N]ATP to give material with near the natural abundance of ^{15}N (0.37%) in the N-6 position of ATP. The observed isotope effects, determined by substitution of mass ratios obtained by isotope ratio mass spectrometry into eq 2, are given in Table I. The isotope effects were determined at two pH values, one at the pH optimum and the other well away from the optimum where catalysis should be more fully rate-limiting, as well as with an alternate slow substrate, 1,5-anhydro-D-glucitol. In each case, the isotope effect could be determined three separate ways either on the basis of the isotopic composition of the residual substrate and the isotopic composition of the starting material (eq 3) or the isotopic composition of the product and starting material (eq 4) or on the basis of isotopic composition of residual substrate and product (eq 2). The agreement of these three methods indicates that no contamination by extraneous materials of mass 29 or 28 is present in the samples unless it is evenly distributed among the starting material, residual substrate, and product. In order to observe such clean results, however, it is necessary to ensure that no ethanol or other material containing ethyl groups gets into the samples, since ethyl radicals, which can be produced in the source of the isotope ratio mass spectrometer, have mass 29.

In the remote-label method, the actual discrimination seen is the product of that caused by the remote-label itself as well as by the isotopic substitution of interest. It is thus necessary to determine the isotope effect at the remote-label position independently by using ATP containing the natural abundance of ^{15}N at this position and no other labeling, and divide this value into that observed with the remote-labeled ATP in order to give the discrimination caused solely by the ^{18}O. The results of these experiments are shown in the bottom part of Table I. The $^{15}(V/K)$ isotope effects were small at both pH 8.2 (1.0004) and pH 5.3 (0.9997) and were used to correct the observed values with the remote-labeled ATP. The final values corrected by eq 5 for incomplete labeling, and by eq 6 for the partial conversion, at pH 5.3, of $MgATP^{2-}$ to $HATP^{3-}$ are shown in Table II.

Discussion

The initial step in an enzymatic reaction is the binding of substrate. With hexokinase, a conformational change is induced by binding of substrate that is thought to exclude most solvent molecules from the active site (Viola & Cleland, 1978). Stripping of the solvent molecules from the nonbridge ^{18}O-labeled γ-phosphate of ATP may produce an equilibrium isotope effect on binding. This isotope effect may be inverse as the result of tightening of the P–O bonds when solvent cannot assist in stabilizing the negatively charged nonbridge oxygens. Since the binding of ATP is at equilibrium, the binding isotope effect will contribute equally to every isotope effect in Tables I and II. This isotope effect can be estimated to have a maximum effect of 0.3% per ^{18}O, based on the magnitude of the isotope effect for the remote-labeled ATP at pH 8.2. On the other hand, the observed value at pH 8.2 could reflect partly an effect on binding and one on phosphoryl transfer.

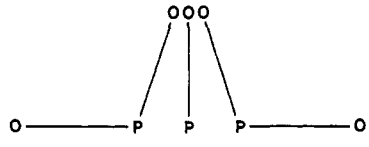

FIGURE 2: Geometry of phosphoryl transfer from phosphate monoesters if the entering and leaving groups stay at a constant distance equal to the sum of single-bond and van der Waals P–O distances. The P–O axial bond order will be 0.06 in the transition state. Note that only the phosphorus undergoes appreciable motion, with the nonbridge oxygens remaining almost stationary with the increased P–O bond order in the transition state compensating for the change from tetrahedral to trigonal geometry.

In any case, as one goes from pH8.2 to 5.3 with glucose, or uses the slow substrate 1,5-anhydroglucitol, catalysis should become more rate-limiting. This certainly occurs as the pH is dropped with glucose, with the isotope effect per ^{18}O changing from 0.1% inverse to 0.3% inverse. With 1,5-anhydroglucitol at pH 8.2, the value is 0.1% more inverse than with glucose. Regardless of how much of the isotope effect at pH 8.2 is caused by a binding isotope effect, making catalysis more rate-limiting gives a more inverse value. This is what is predicted for a dissociative type of mechanism and is what was observed with alkaline phosphatase by Weiss and Cleland (1989). Ab initio calculations of the equilibrium ^{18}O isotope effect for metaphosphate formation from a phosphomonoester give a value of 0.998 per ^{18}O (J. P. Jones, unpublished results). We thus conclude that hexokinase has a dissociative transition state for phosphoryl transfer.[2] The reason for the slow reaction with 6-thioglucose and 6-aminoglucose is not clear; however, if these molecules form either weaker or nonproductive hydrogen bonds to the aspartate group located in the active site of hexokinase, the slow rates may result from failure to induce the proper conformational change which leads to catalysis.

The reason that phosphoryl transfer from phosphate monoesters is dissociative may simply reflect the geometry involved and the fact that the entering and leaving groups maintain a constant distance between them during the transfer. Assuming that the entering group and the phosphorus are in van der Waals contact as the reaction begins, this P–O distance is ≈3.3 Å, while the single bond to the leaving group is ≈1.6 Å. If the phosphorus is halfway between the entering and leaving groups in the transition state and the overall O–P–O distance does not change, each P–O distance in the transition state will be ≈2.45 Å. This situation is diagrammed in Figure 2. According to Pauling's rule, which states that the interatomic distance is the single-bond distance minus 0.3 ln (bond order), this model gives 0.06 as the P–O bond order in the transition state. This is clearly a dissociative transition state. Even if there is compression of the reaction coordinate to a bond order of 0.1 in the transition state, the O–P–O distance only has to decrease from 4.9 to 4.6 Å. To achieve a bond order ot 0.5 to the entering and leaving groups, the O–P–O distance would have to decrease to 3.6 Å, while for a more associative transition state with 0.8 bond order to the two groups in the transition state, the distance must be 3.33 Å.

It is thus tempting to postulate that phosphoryl transfer for monoesters does not involve appreciable compression of the reaction coordinate but does not involve expansion of it either. Phosphate di- and triester hydrolyses require more compression of the reaction coordinate and thus more associative transition states. Some compression of the reaction coordinate by an enzyme would probably increase the reaction rate for a monoester, and this may be one method of catalysis used by enzymes. The observed isotope effects to date for hexokinase and alkaline phosphatase suggest, however, that the axial bond orders in the transition state are still small.

[2] Both a dissociative mechanism with a metaphosphate intermediate and a concerted mechanism with an exploded transition state with low axial bond order would give inverse secondary ^{18}O isotope effects. These experiments rule out transition states with associative character.

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