Catalytic and Allosteric Mechanism of AMP Nucleosidase from Primary, β-Secondary, and Multiple Heavy Atom Kinetic Isotope Effects†

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ABSTRACT: Adenosine 5′-phosphate was synthesized with specific heavy atom substitutions to permit measurement of V/K kinetic isotope effects for the N-glycohydrolase activity of the allosteric AMP nucleosidase and the acid-catalyzed solvolysis of these compounds. The effects of allosteric activation on the kinetic isotope effects together with the kinetic mechanism of AMP nucleosidase [DeWolf, W. E., Jr., Emig, F. A., & Schramm, V. L. (1986) *Biochemistry 25*, 4132–4140] indicate that the kinetic isotope effects are fully expressed. Comparison of individual primary and secondary kinetic isotope effects with combined isotope effects and the isotope effect of the reverse reaction indicated that kinetic isotope effects in AMP nucleosidase arise from a single step in the reaction mechanism. Under these conditions, kinetic isotope effects can be used to interpret transition-state structure for AMP nucleosidase. Changes in kinetic isotope effects occurred as a function of allosteric activator, demonstrating that allosteric activation alters transition-state structure for AMP nucleosidase. Kinetic isotope effects, expressed as [V/K(normal isotope)]/[V/K(heavy isotope)], were observed with [2′-^{2}H]AMP (1.061 ± 0.002), [9-^{15}N]AMP (1.030 ± 0.003), [1′-^{2}H]AMP (1.045 ± 0.002), and [1′-^{14}C]AMP (1.035 ± 0.002) when hydrolyzed by AMP nucleosidase in the absence of MgATP. Addition of MgATP altered the [2′-^{2}H]AMP effect (1.043 ± 0.002) and the [1′-^{2}H]AMP effect (1.030 ± 0.003) and caused a smaller decrease of the ^{14}C and ^{15}N effects. Multiple heavy atom substitutions into AMP caused an increase in observed isotope effects to 1.084 ± 0.004 for [1′-^{2}H,1′-^{14}C]AMP and to 1.058 ± 0.002 for [9-^{15}N,1′-^{14}C]AMP with the enzyme in the absence of ATP. The primary ^{15}N kinetic isotope effects were the same for acid- and enzyme-catalyzed hydrolysis while all of the secondary isotope effects were smaller for the enzyme-catalyzed hydrolysis. The secondary ^{3}H isotope effects (with [1′-^{3}H]AMP or [1-^{3}H]ribose 5-phosphate) were approximately the same for the forward (1.047 ± 0.002) and reverse (1.06 ± 0.01) reactions, establishing an equilibrium isotope effect near unity and confirming the intrinsic nature of the isotope effects. The isotope effects indicate that the transition-state complex of AMP nucleosidase is oxycarbonium-like with hindered out-of-plane motion of the C1′ hydrogen. In the presence of allosteric activator the bonding to C1′ of ribose 5-phosphate in the transition state is unchanged, but the out-of-plane bending of the C1′ and C2′ hydrogens is further restricted.

The purpose of this study was to determine the transition-state structure for the acid-catalyzed hydrolysis of the N-glycosidic bond of AMP and to compare this structure to the transition state for the enzymatic hydrolysis of AMP. The enzyme selected for these studies, AMP nucleosidase (EC 3.2.2.4), has the additional property of being activated by MgATP, an allosteric activator, which increases the enzymatic turnover number by a factor of 10^2–10^3 depending on the substrate concentration (Schramm, 1974; DeWolf et al., 1979). Quantitative analysis of transition-state structures for enzymatic reactions requires the measurement of a sufficient number of kinetic isotope effects to establish transition-state geometry, as in similar studies for chemical reactions [e.g., Melander and Saunders (1980)]. In addition, factors that can suppress kinetic isotope effects in enzymatic reactions must be eliminated or quantitated [e.g., Cleland (1982)]. These include a commitment to catalysis for substrate, multiple intermediates in the reaction mechanism that have similar activation energies (Northrop, 1981), and other steps that may obscure the intrinsic kinetic isotope effect.

Initial studies with AMP nucleosidase from *Azotobacter vinelandii* indicated that significant primary ^{14}C and secondary ^{3}H kinetic isotope effects were expressed in both the presence and absence of MgATP, but these were not established as intrinsic isotope effects (Parkin & Schramm, 1984). More recent chemical and kinetic studies have established a rapid-equilibrium random, concerted mechanism for AMP nucleosidase with no commitment to catalysis for the enzyme–AMP complex (DeWolf et al., 1986). These results, together with the additional kinetic isotope effects reported here, establish the intrinsic nature of the kinetic isotope effects and establish that a single step in the mechanism gives rise to these isotope effects. In the following paper (Mentch et al., 1987) these effects are used to calculate specific transition-state structures for hydrolysis of the N-glycosidic bond of AMP. The results of these studies provide the first direct evidence that allosteric activation can alter transition-state structure. For AMP nucleosidase, this is clearly a consequence of an allosteric transition since the catalytic and allosteric sites are separated by greater than 20 Å (DeWolf et al., 1980).

Experimental Procedures

Materials

Carrier-free [6-^{14}C]glucose, [2-^{14}C]glucose, and [6-^{3}H]-glucose were purchased from Amersham. Enzymes were

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Table I: Kinetic Isotope Effects for Acid and Enzymatic Hydrolysis of AMP

labeled substrates	position of heavy atom	kinetic isotope effect[a]		
		acid catalyzed	enzyme catalyzed	
			no ATP	+MgATP
[1′-^{2}H,5′-^{3}H]AMP + [5′-^{14}C]AMP	α-secondary ^{2}H	1.123 ± 0.004 (6)	1.045 ± 0.002 (9)[b]	1.030 ± 0.003 (3)
[1′-^{3}H]AMP + [5′-^{14}C]AMP	α-secondary ^{3}H	1.216 ± 0.004 (3)	1.069 ± 0.003 (3)	1.047 ± 0.002 (3)
[1-^{3}H]R5P[c] + [5-^{14}C]R5P, reverse reaction	α-secondary ^{3}H			1.06 ± 0.01 (3)
[1′-^{14}C]AMP + [5′-^{3}H]AMP	primary ^{14}C	1.044 ± 0.003 (3)	1.035 ± 0.002 (9)	1.032 ± 0.002 (9)
[2′-^{2}H,5′-^{3}H]AMP + [5′-^{14}C]AMP	β-secondary ^{2}H	1.077 ± 0.002 (3)	1.061 ± 0.002 (3)	1.043 ± 0.002 (3)
[9-^{15}N,5′-^{14}C]AMP + [5′-^{3}H]AMP	primary ^{15}N	1.030 ± 0.002 (3)	1.030 ± 0.003 (3)	1.025 ± 0.002 (3)
[1′-^{2}H,1′-^{14}C]AMP + [5′-^{3}H]AMP	combined primary ^{14}C and α-secondary ^{2}H	1.158 ± 0.004 (6)	1.084 ± 0.004 (3)	1.058 ± 0.002 (3)
[9-^{15}N,1′-^{14}C]AMP + [5′-^{3}H]AMP	combined primary ^{14}C and primary ^{15}N	1.064 ± 0.003 (3)	1.059 ± 0.002 (3)	1.048 ± 0.003 (3)
[5′-^{3}H]AMP + [5′-^{14}C]AMP	control[d]	1.006 ± 0.002 (6)	1.007 ± 0.003 (6)	1.006 ± 1.002 (6)

[a] Kinetic isotope effects are expressed as follows: $[V/K(\text{normal isotope})]/[V/K(\text{heavy isotope})]$ = kinetic isotope effect. The values reported in the table are those obtained by direct calculations using eq 1 and 2 under Methods. Corrections are made for the control effect of ^{3}H at the 5′-position. The number in parentheses is the number of isotope effect determinations. [b] Measured at three different substrate concentrations as indicated in the text. [c] Ribose 5-phosphate is abbreviated as R5P. [d] Control kinetic isotope effect calculated as (^{14}C/^{3}H ratio at 20% substrate hydrolysis)/(^{14}C/^{3}H ratio at 100% substrate hydrolysis).

purchased from P-L Biochemicals or Sigma Chemical Co. and were the highest purity (by specific enzymatic activity) available. Adenine phosphoribosyltransferase was partially purified from *Escherichia coli* (Parkin et al., 1984) by a modification of the method described by Hochstadt (1978). Phosphoribosylpyrophosphate synthetase was the generous gift of Dr. Robert Switzer, University of Illinois, Urbana. [^{15}N]Ammonium chloride (97% isotope substitution) was purchased from Merck Corp., and 5-amino-4,6-dichloropyrimidine, 4-chloro-5,6-diaminopurine, 6-chloropurine, and diethoxymethyl acetate were purchased from Aldrich Chemical Co. Nucleotides and adenine were products of P-L Biochemicals. Labeled adenosine 5′-monophosphates [5′-^{14}C]-AMP, [1′-^{3}H]AMP, [1′-^{14}C]AMP, and [5′-^{3}H]AMP were prepared as previously described (Parkin et al., 1984). AMP nucleosidase was purified from *A. vinelandii* as previously described (Schramm & Leung, 1978).

Methods

Synthesis of Labeled AMP. Adenosine 5′-monophosphates were synthesized from precursors containing the desired heavy atoms in the appropriate positions. The enzymatic methods for several of these syntheses have been described previously (Parkin et al., 1984). The synthetic approach was to incorporate ^{3}H or ^{14}C into the ribose 5-phosphate portion of AMP to permit analysis of products by the ^{3}H/^{14}C ratios. In some cases the radioactive label also served as the heavy atom in isotopically sensitive positions. In other cases, the radioactive label was remote from the heavy atom substitution. For example, when ^{2}H or ^{15}N was used as the heavy atom substitution, the radioactive labels were placed in the 5′-position of AMP. Table I lists the compounds synthesized and used in this study.

Synthesis of [1′-^{2}H,5′-^{14}C]AMP, [1′-^{2}H,1′-^{14}C]AMP, and [1′-^{2}H,5′-^{3}H]AMP. Incorporation of deuterium into the 1′-position of AMP was accomplished with ^2H_2O as solvent for phosphoglucose isomerase catalyzed exchange into the desired position. The radioactive labels were incorporated from ^{3}H or ^{14}C glucose as the precursor for the ribosyl group of AMP.

Carrier-free [2-^{14}C]glucose or [6-^{14}C]glucose (55 mCi/mmol) or [6-^{3}H]glucose (20 Ci/mmol) was dried under vacuum. The initial reaction mixture was added to the labeled glucose to give 50 mM glycylglycine or triethanolamine, pH 7.5, 0.1 mM ATP, 2 mM phosphoenolpyruvate, 1 mM $MgCl_2$, 5 units/mL pyruvate kinase, 0.4 units/mL hexokinase, and 1 mM labeled glucose with a specific radioactivity of 20–55 mCi/mmol. Enzymes were used as supplied by the manufacturer without prior dialysis. Hexokinase was added to start the reaction. Reaction mixtures (1 mL) were incubated at 37 °C for approximately 30 min until the glucose had been phosphorylated. The reaction was monitored by placing 1 μL of the reaction mixture on a 0.5 × 4 cm column of DEAE-Sephadex A-25, acetate form, and eluting with 20 mM acetic acid. Under these conditions glucose is eluted and glucose 6-phosphate is retained on the column. The mixture was evaporated to dryness and dissolved in ^2H_2O (100 atom %) to give 2.5 mL. Phosphoglucose isomerase (36 units/mL) was added, and the reaction mixture was incubated overnight at room temperature. The reaction mixture was diluted with nicotinamide adenine dinucleotide phosphate (NADP) in ^2H_2O to give a final concentration of 1 mM hexose and 5 mM NADP. Glucose-6-phosphate dehydrogenase (0.4 unit/mL) and 6-phosphogluconate dehydrogenase (0.1 unit/mL) were added, and the reaction mixture was incubated overnight at 37 °C. The mixture was evaporated to dryness under reduced pressure and reconstituted to the same volume with buffer in H_2O to give additional concentrations of 50 mM potassium phosphate, pH 7.5, 20 mM phosphoenolpyruvate, 10 mM $MgCl_2$, 3.2 mM adenine, 0.5 unit/mL myokinase, 2.5 units/mL pyruvate kinase, and 5 units/mL phosphoribose isomerase. The reaction mixture was incubated for 2 h at 37 °C, followed by the addition of 0.07 unit/mL of phosphoribosylpyrophosphate synthetase and 0.05 unit/mL adenine phosphoribosyltransferase. The reaction mixture was then incubated at 37 °C overnight, placed in boiling water for 3 min, and cooled on ice. The resulting ATP was converted to AMP by the addition of glucose to a final concentration of 6 mM, 25 units/mL hexokinase, and 200 units/mL myokinase. The mixture was incubated at 37 °C for 30 min and in boiling H_2O for 3 min. The labeled AMP was purified by gel filtration, DEAE-Sephadex chromatography, and when necessary, high-performance liquid chromatography (Parkin et al., 1984). Overall yield of purified AMP from labeled glucose varied from 40% to 90%. The enzymatic synthesis of these compounds is outlined in Figure 1 and the legend to Figure 1.

Synthesis of [2′-^{2}H,5′-^{3}H]AMP. Reaction mixtures contained 50 mM potassium phosphate, pH 7.5, 50 mM glycylglycine, pH 7.5, 3.2 mM adenine, 10 mM $MgCl_2$, 0.1 mM ATP, 5 mM NADP, 20 mM phosphoenolpyruvate, 0.4 unit/mL hexokinase, 2 units/mL adenylate kinase, 5 units/mL pyruvate kinase, 0.4 unit/mL glucose-6-phosphate dehydrogenase, 0.3 unit/mL 6-phosphogluconate dehydrogenase, 5 units/mL phosphoribose isomerase, 0.05 unit/mL phos-

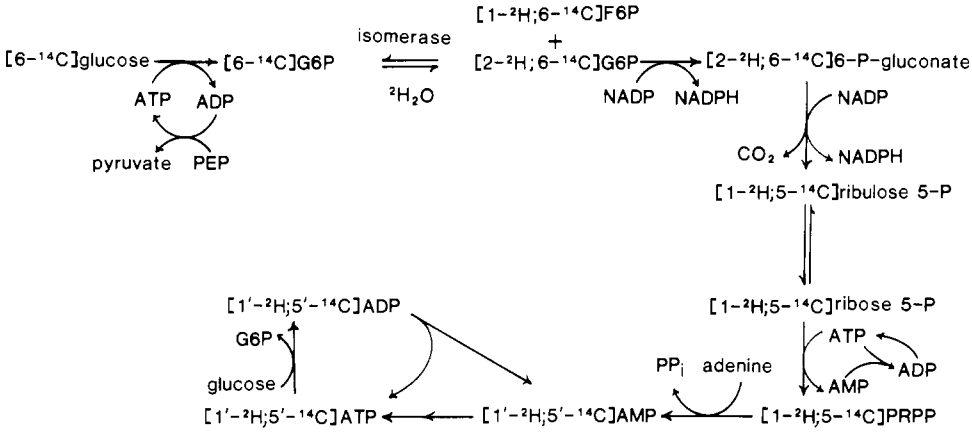

FIGURE 1: Synthesis of AMP labeled with ^2H in the 1'-position. The procedure is given for synthesis of [1'-^{2}H,5'-^{14}C]AMP. Substitution of [2-^{14}C]glucose as starting material yields [1'-^{2}H,1'-^{14}C]AMP. Likewise, use of [6'-^{3}H]glucose in the procedure yields [1'-^{2}H,5'-^{3}H]AMP. Glucose 6-phosphate is formed in H_2O during the first step. In the second step ^2H is exchanged from the ^2H_2O solvent into glucose 6-phosphate by hexosephosphate isomerase. The third step converts glucose 6-phosphate to the pentose phosphates, and the fourth step completes the conversion of pentose phosphates to labeled ATP. Labeled AMP is converted to ATP by the combined actions of adenylate kinase and pyruvate kinase. The final step involves conversion of the labeled ATP to AMP by the combined reactions of hexokinase and adenylate kinase. Details of the procedure are given in the text. Abbreviations: G6P, glucose 6-phosphate; PEP, phosphoenolpyruvate; isomerase, hexosephosphate isomerase; F6P, fructose 6-phosphate; PRPP, 5-phosphoribosyl 1-pyrophosphate.

phoribosylpyrophosphate synthetase, 0.05 unit/mL adenine phosphoribosyltransferase, and 1 mM labeled [6-^{3}H]glucose (20 mCi/mmol). All reagents except enzymes, potassium phosphate, and adenine were evaporated to dryness and dissolved in ^2H_2O. Potassium phosphate and adenine were dried separately and dissolved in ^2H_2O. All enzymes except phosphoribosylpyrophosphate synthetase and adenine phosphoribosyltransferase were combined as the ammonium sulfate precipitates and centrifuged to a small pellet, and the walls of the tube were carefully dried. The other components were then added, followed by 3-h incubation at 37 °C. Phosphoribosylpyrophosphate synthetase and adenine phosphoribosyltransferase were added in less than 0.5% of the final volume. The final addition of enzymes was in H_2O. The completed reaction mixture was incubated overnight at 37 °C. The resulting ATP was converted to AMP and purified as described above. The overall yield of purified, labeled AMP from glucose starting material was from 40% to 90%. The reaction outline for synthesis of this compound is given in Figure 2.

Synthesis of [9-^{15}N,5'-^{3}H]AMP and [9-^{15}N,1'-^{14}C]AMP. Synthesis of [9-^{15}N]adenine followed a modified procedure of Sethi et al. (1982), using 5-amino-4,6-dichloropyrimidine as starting material. The ^{15}N was contributed by [^{15}N]ammonia of 97% isotopic enrichment. [^{15}N]Ammonia was generated from [^{15}N]ammonium chloride by the addition of 0.5 g of $^{15}NH_4Cl$ to 3 g of NaOH pellets. The resulting $^{15}NH_3$ was passed through a small drying tube containing additional NaOH pellets and dissolved in a solution of 8 mL of anhydrous ethanol containing 0.15 g of 5-amino-4,6-dichloropyrimidine, cooled to dry ice–ethanol temperature. The mixture (in a glass tube) was placed in a Parr reaction bomb and heated to 133 °C for 17 h. The solution was cooled to room temperature, evaporated to dryness, and dissolved in the minimum volume of H_2O at 90 °C. The solution was filtered while hot and the filtrate placed on ice until crystals were formed. The product (85 mg, 64% yield) was 4-chloro-5,6-diaminopyrimidine by melting point, ultraviolet absorbance spectrum, and high-performance liquid chromatography, with authentic 4-chloro-5,6-diaminopyrimidine as standard. 4-Chloro-5,6-diaminopyrimidine was converted to 6-chloropurine by dissolving 45 mg of 4-chloro-5,6-diaminopurine in 3 mL of diethoxymethyl acetate in a glass tube, sealing in a Parr reaction bomb,

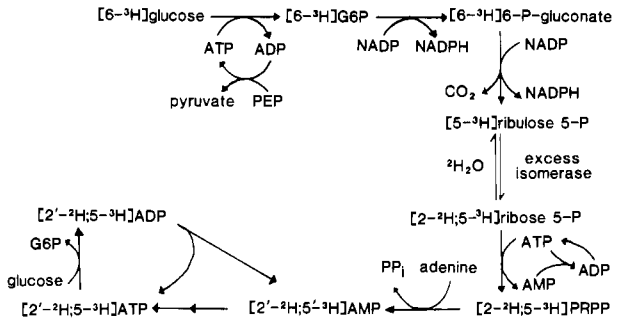

FIGURE 2: Synthesis of AMP labeled with ^2H in the 2'-position. The procedure for the synthesis of [2'-^{2}H,5'-^{3}H]AMP is given. The first incubation converts glucose 6-phosphate to the pentose phosphates and equilibrates the hydrogen at C2 with solvent ^2H. The second step converts labeled ribose 5-phosphate to labeled ATP. In the final step, labeled ATP is converted to AMP by the combined actions of hexokinase and adenylate kinase. Only the ^2H label that is incorporated into the final product is indicated in the figure. Transient label (^2H that is incorporated at one step and lost in a subsequent step) is not indicated. Details of the procedure are given in the text. Abbreviations are given in the legend to Figure 1, except that isomerase represents phosphoribose isomerase.

and heating for 7 h at 114 °C. The solution was evaporated to dryness and the residue extracted in methanol–H_2O, 1:1. The sample (0.8 mL) was applied to a 1 × 25 cm Beckman ODS 5-μm reverse-phase high-performance liquid chromatography column previously equilibrated with 50% methanol and eluted with the same solution. The fractions containing 6-chloropurine were identified by the ultraviolet absorbance spectrum and were pooled. The product (48% yield) coeluted with authentic 6-chloropurine by high-performance liquid chromatography and exhibited the expected ultraviolet absorbance spectra at pH 5 and 11. 6-Chloropurine was converted to adenine by dissolving 45 mg of 6-chloropurine in 5 mL of anhydrous ethanol and cooling in dry ice–ethanol. The solution was saturated with NH_3 by bubbling dry NH_3 gas into the solution as described above. The mixture, in a glass tube, was sealed in a Parr reaction vessel and incubated for 19 h at 135 °C. The solution was dried, extracted into 50% ethanol, dried, and dissolved in H_2O. The product [9-^{15}N]-adenine gave a single peak on high-performance liquid chromatography, coeluted with authentic adenine, and gave the expected ultraviolet absorbance spectra at pH 2 and 11.

Overall yield of [9-^{15}N]adenine from 4,6-dichloro-5-aminopyrimidine was 20%. For the synthesis of [9-^{15}N,5′-^{3}H]AMP, the reaction used [9-^{15}N]adenine and [6-^{3}H]glucose as precursors. Synthesis of [9-^{15}N,1′-^{14}C]AMP used [9-^{15}N]adenine and [2-^{14}C]glucose as precursors. Conversion of these precursors to the labeled AMP molecules and purification of the AMP were as previously described (Parkin et al., 1984).

Analysis of Labeled AMP. Incorporation of ^{3}H or ^{14}C into the appropriate positions of AMP by the enzymatic synthetic scheme used here has been previously documented by degradation of the sugar and analysis of products for the presence of radioactivity in specific positions (Parkin et al., 1984). Incorporation of ^{2}H into the 1′ and 2′ positions of AMP was analyzed by proton NMR analysis of the purified AMP. This analysis was complicated by the dilution with unlabeled ATP, used to initiate the reactions (see Figures 1 and 2). Extent of ^{2}H incorporation was calculated from the proton NMR spectrum of AMP and the specific radioactivity of AMP compared to the specific radioactivity of the starting glucose. Analysis by NMR indicated that the enzymatic biosynthesis was specific, with no detectable ^{2}H appearing in the 2′-position when 1′ substitution was desired and no detectable incorporation of ^{2}H in the 1′-position when incorporation into the 2′-position was desired. The proton NMR spectrum of the C1′ proton allowed direct determination of the extent of ^{2}H substitution at C1′ by integration of the area under the proton peaks. The presence of ^{2}H at C2′ was quantitated by loss of splitting of the C1′ doublet as ^{1}H is replaced by ^{2}H at C2′.

The position of ^{15}N incorporation into adenine was determined by ^{15}N NMR. The ^{15}N NMR spectrum of putative [9-^{15}N]adenine gave a single ^{15}N resonance at the position expected for [9-^{15}N]adenine. The [9-^{15}N]adenine and [9-^{15}N]AMP were assumed to have the same isotopic composition as the $^{15}NH_4Cl$ starting material. Control incubation mixtures containing all of the ingredients for synthesis of [9-^{15}N]AMP, but without adenine, were incubated and assayed for the appearance of adenine, since the only source for isotopic dilution of [9-^{15}N]adenine would be from adenine generated from ATP. These experiments demonstrated that no adenine is formed from ATP under the conditions used for [9-^{15}N]AMP synthesis.

Determination of Kinetic Isotope Effects. Kinetic isotope effects for hydrolysis of AMP were determined by measuring the ratio of $^{3}H/^{14}C$ in ribose 5-phosphate formed by the hydrolysis of labeled AMP mixtures to adenine and ribose 5-phosphate. Unreacted AMP, adenine, and ATP were separated from ribose 5-phosphate by chromatography on small charcoal columns that had been previously washed with unlabeled ribose 5-phosphate. Elution of the labeled ribose 5-phosphate was with 10 mM ribose 5-phosphate. Samples of 0.50 g were diluted with 0.50 mL of H_2O and to 10.0 mL with Liquiscint (National Diagnostics), followed by analysis for $^{3}H/^{14}C$ in a dual-channel scintillation spectrometer. In most cases the isotope effect was determined by comparison of the $^{3}H/^{14}C$ ratio of ribose 5-phosphate generated during the first 10–30% of AMP hydrolysis relative to the $^{3}H/^{14}C$ ratio in ribose 5-phosphate following conversion of 99 ± 1% of the AMP to ribose 5-phosphate. Labeled AMP mixtures were analyzed for the ratio of $^{3}H/^{14}C$ in the AMP compared to the ratio in the ribose 5-phosphate at 99 ± 1% hydrolysis. If these ratios were not within the standard error of the measurement (±0.2%), the labeled AMP was further purified. The remainder of the techniques used for measurement of the kinetic isotope effects were the same as described in Parkin et al. (1984).

Kinetic isotope effects for the synthesis of AMP from adenine and ribose 5-phosphate were determined by measuring the ratio of $^{3}H/^{14}C$ in AMP formed in the reverse reaction using [5-^{14}C]ribose 5-phosphate and [1-^{3}H]ribose 5-phosphate as substrates. A mixture of [1′-^{3}H]AMP and [5′-^{14}C]AMP was hydrolyzed completely to adenine and ribose 5-phosphate in a reaction mixture of 0.1 mL containing 2 mM AMP, 0.5 mM ATP, 1 mM $MgCl_2$, 0.1 M triethanolamine hydrochloride, pH 8.0, and 0.1 unit of AMP nucleosidase. The mixture was freeze-dried and reconstituted to 0.1 mL containing 20 mM adenine, 20 mM ribose 5-phosphate, 40 mM phosphoenolpyruvate, 50 mM KCl, 0.5 mM ATP, 1 mM $MgCl_2$, 0.1 M triethanolamine hydrochloride, pH 8.0, 200 units of pyruvate kinase, 200 units of adenylate kinase, and 78 units of AMP nucleosidase. Under these conditions, approximately 20% of the ribose 5-phosphate was converted to ATP in 1 h. The reaction was stopped by boiling for 5 min. The mixture was then diluted to 0.5 mL and made 10 mM in glucose, and 5 units of hexokinase and 5 units of adenylate kinase were added and incubated for 1 h at 30 °C to convert the labeled ATP to AMP. The reaction mixture was diluted to 1.0 mL with 1 M acetic acid, divided into three portions, and applied to three DEAE-Sephadex A-50 columns in Pasteur pipets, which were equilibrated with 0.5 M acetic acid. Adenine was eluted with 0.5 M acetic acid, labeled AMP was eluted with 1 M acetic acid, and ribose 5-phosphate remained bound to the column. Fractions containing the labeled AMP were pooled, reduced to 1.0 mL under a nitrogen stream, and freeze-dried. The AMP was resuspended in 1.0 mL of H_2O, mixed with 10 mL of scintillation fluid, and counted for the $^{14}C/^{3}H$ ratio as described above. The $^{14}C/^{3}H$ ratio was compared to that of the original AMP used to generate the labeled ribose 5-phosphate. All samples contained equivalent amounts of unlabeled AMP. Tritium and ^{14}C standards also contained an equivalent concentration of AMP.

The general formula used for calculation of kinetic isotope effects is

$$\text{obsd isotope effect} = \frac{\text{radioisotope representing natural isotope}}{\text{radioisotope representing heavy isotope}}\ (10\text{–}30\%\ \text{hydrolysis}) \Big/ \frac{\text{radioisotope representing natural isotope}}{\text{radioisotope representing heavy isotope}}\ (100\%\ \text{hydrolysis}) \quad (1)$$

The observed kinetic isotope effects were corrected to 0% hydrolysis with the expression

$$\text{actual isotope effect} = \frac{\ln\,[1 - (\text{fraction hydrolyzed})(\text{obsd isotope effect})]}{\ln\,(1 - \text{fraction hydrolyzed})} \quad (2)$$

For example, measurement of the ^{15}N kinetic isotope effect used a mixture of [9-^{15}N,5′-^{14}C]AMP and [5′-^{3}H]AMP. The observed isotope effect calculation using eq 1 is

$$\text{obsd isotope effect} = \frac{(^{3}H/^{14}C)\ (10\text{–}30\%\ \text{hydrolysis})}{(^{3}H/^{14}C)\ (100\%\ \text{hydrolysis})}$$

Other isotope effects, for example, the β-secondary ^{2}H effect with a mixture of [2′-^{2}H,5′-^{3}H]AMP and [5′-^{14}C]AMP, were calculated with the opposite $^{14}C/^{3}H$ ratio:

$$\text{obsd isotope effect} = \frac{(^{14}C/^{3}H)\ (10\text{–}30\%\ \text{hydrolysis})}{(^{14}C/^{3}H)\ (100\%\ \text{hydrolysis})}$$

RESULTS

Primary ^{14}C Kinetic Isotope Effect. The primary ^{14}C isotope effect was measured by using a mixture of [1′-^{14}C]AMP and [5′-^{3}H]AMP as substrates. The enzymatic hydrolysis was repeated several times in the absence and presence of MgATP to establish if the primary ^{14}C isotope effect is independent of allosteric activator. The average value of nine determinations in the absence of MgATP was 1.035 ± 0.002. In the presence of 0.5 mM MgATP the average kinetic isotope effect was 1.032 ± 0.002. These results confirm the earlier report that the primary ^{14}C kinetic isotope does not change significantly as the reaction rate is increased approximately 200-fold by the presence of allosteric activator (Parkin & Schramm, 1984).

The acid-catalyzed hydrolysis of [1-^{14}C]AMP had been previously reported to give a kinetic isotope effect of 1.049 ± 0.009 (Parkin et al., 1984). Because of the relatively large error of this measurement, the experiment was repeated to more accurately establish this value. The results of these experiments gave a primary ^{14}C heavy isotope effect of 1.044 ± 0.003, as summarized in Table I.

β-Deuterium Kinetic Isotope Effects. Hydrolysis of a mixture of [2′-^{2}H,5′-^{3}H]AMP and [5′-^{14}C]AMP provides a method for measuring the effect of β-^{2}H substitution on N-glycosidic bond cleavage. Labeled AMP was converted to adenine and ribose 5′-phosphate by incubation at 50 °C in 0.1 M HCl or by AMP nucleosidase. The kinetic isotope effect due to substitution of ^{2}H at the 2′-position of AMP by acid-catalyzed solvolysis at 50 °C was 1.077 ± 0.002 for $^{1}H/^{2}H$. Under these conditions of hydrolysis, the pseudo-first-order rate constant for AMP hydrolysis is approximately 5×10^{-6} s^{-1}.

The β-secondary ^{2}H kinetic isotope effect was also measured with AMP nucleosidase as the catalyst. In the absence of MgATP, where the enzymatic turnover number is approximately 0.2 s^{-1}, the kinetic isotope effect was 1.061 ± 0.002. Addition of the allosteric activator MgATP increased the enzymatic turnover number to approximately 60 s^{-1} and decreased the $^{1}H/^{2}H$ β-secondary isotope effect to 1.043 ± 0.002. These data are summarized in Table I.

α-Deuterium Kinetic Isotope Effects. Hydrolysis of [1′-^{2}H,5′-^{3}H]AMP and [5′-^{14}C]AMP and determination of the $^{3}H/^{14}C$ ratio permit estimation of the α-secondary deuterium kinetic isotope effect. The labeled AMP mixture was hydrolyzed in 0.1 M HCl or by enzymatic hydrolysis as described above. Acid-catalyzed solvolysis gave an α-secondary ^{2}H kinetic effect of 1.123 ± 0.004.

Hydrolysis of the same nucleotide pair with AMP nucleosidase in the absence of MgATP gave an isotope effect of 1.045 ± 0.002. In the presence of allosteric activator, the isotope effect decreased to 1.030 ± 0.003. These values agree with the α-^{3}H kinetic isotope effects previously measured for AMP nucleosidase and the acid-catalyzed hydrolysis of AMP (Parkin et al., 1984; Parkin & Schramm, 1984). Values of the α-^{3}H effects are included in Table I for purposes of comparison.

The α-deuterium kinetic isotope effect for hydrolysis of AMP by AMP nucleosidase was also measured as a function of substrate concentration in the absence of allosteric activator. The kinetic isotope effect was constant with a value of 1.045 ± 0.002 as total AMP concentration was varied from 0.2 to 9.2 mM. These concentrations cover the range from $1.9K_m$ to $88K_m$ for AMP nucleosidase.

Primary ^{15}N Kinetic Isotope Effect. Hydrolysis of a mixture of [9-^{15}N,5′-^{14}C]AMP and [5′-^{3}H]AMP followed by determination of the $^{3}H/^{14}C$ ratio in the ribose 5-phosphate permits determination of the primary ^{15}N isotope effect for hydrolysis of the N-glycosidic bond of AMP. In the presence of 0.1 M HCl at 50 °C, the acid-catalyzed solvolysis gave a kinetic isotope effect of 1.030 ± 0.002. Hydrolysis of the same nucleotide pair with AMP nucleosidase in the absence of allosteric activator gave the same isotope effect of 1.030 ± 0.003. The addition of 0.5 mM MgATP as allosteric activator increased the rate of hydrolysis approximately 200-fold but caused only a small change in the primary ^{15}N isotope effect to 1.025 ± 0.002 (Table I).

Combined Primary ^{14}C and α-^{2}H Kinetic Isotope Effects. Hydrolysis of a mixture of [1′-^{2}H,1′-^{14}C]AMP and [5′-^{3}H]-AMP followed by determination of the $^{3}H/^{14}C$ ratio in the ribose 5-phosphate permits determination of the combined primary and secondary isotope effects. Acid-catalyzed solvolysis in the presence of 0.1 M HCl at 50 °C gave a combined isotope effect of 1.158 ± 0.004. Hydrolysis by AMP nucleosidase in the absence of allosteric activation gave a combined kinetic isotope effect of 1.084 ± 0.004, which decreased to 1.058 ± 0.002 when the enzyme catalyzed the reaction in the presence of 0.5 mM MgATP. These values should be compared to the values for the individual primary ^{14}C and the α-^{2}H secondary kinetic isotope effects that are given in Table I. In all cases, the presence of the α-^{2}H substitution increased the magnitude of the primary ^{14}C isotope effect.

Combined Primary ^{14}C and Primary ^{15}N Kinetic Isotope Effects. Hydrolysis of a mixture of [9-^{15}N,1′-^{14}C]AMP and [5′-^{3}H]AMP followed by determination of the $^{3}H/^{14}C$ ratio in the ribose 5-phosphate permits determination of the combined primary ^{14}C and ^{15}N kinetic isotope effects. Acid-catalyzed hydrolysis of this substrate pair resulted in a kinetic isotope effect of 1.064 ± 0.003, which is slightly greater than the effect with enzyme-catalyzed hydrolysis in the absence of MgATP (Table I). Thus, hydrolysis of this mixture by AMP nucleosidase in the absence of allosteric activation gave a combined kinetic isotope effect of 1.059 ± 0.002. In the presence of 0.5 mM MgATP, the kinetic isotope effect decreased to 1.048 ± 0.003. The multiple kinetic isotope effects should be compared to the individual primary ^{14}C and ^{15}N kinetic isotope effects given in Table I. The combined kinetic isotope effect is approximately equal to the product of the individual heavy atom primary isotope effects for each of the three experimental conditions.

α-Tritium Secondary Isotope Effect for the Reverse Reaction of AMP Nucleosidase. Incubation of AMP nucleosidase with a mixture of [1-^{3}H]ribose 5-phosphate, [5-^{14}C]-ribose 5-phosphate, and adenine under conditions that rapidly convert product AMP to ATP permits study of the reverse reaction. The average kinetic isotope effect for three such determinations was 1.06 ± 0.01, close to the value of the related kinetic isotope effect of 1.047 ± 0.002, with [1′-^{3}H]-AMP for the hydrolytic reaction (Table I). The error associated with the isotope effect in the reverse reaction is due to the unfavorable kinetic constants for ribose 5-phosphate and the increased manipulation to isolate product AMP.

Measured Kinetic Isotope Effects with ^{3}H and ^{14}C in Isotopically "Insensitive" Positions. The validity of kinetic isotope effects depends on the ability to discriminate between small changes in reactivity of the compounds with labels at or adjacent to the glycosidic bond compared to compounds with labels at the 5′-position of AMP. The control experiment for kinetic isotope effects is the analysis of ^{3}H and ^{14}C ratios at ~20% and 100% hydrolysis where ^{3}H and ^{14}C are both in the 5′-position. A mixture of [5′-^{14}C]AMP and [5′-^{3}H]AMP was hydrolyzed by acid and by AMP nucleosidase both in the

presence and in the absence of MgATP. The results of these experiments are summarized in Table I. All experimental conditions gave a small but reproducible [5′-^{3}H]AMP kinetic isotope effect of 1.006 ± 0.002 (the presence of 5′-^{3}H decreases the reaction rate). Since the value of the kinetic isotope effect did not vary for acid- or enzyme-catalyzed hydrolysis, the eighteen control experiments were averaged to give a [5′-^{3}H]AMP kinetic isotope effect of 1.006 ± 0.001. Since this standard error is small compared to the values for individual kinetic isotope effect experiments, the measured isotope effects were corrected by the factor of 0.006. The correction increased the observed kinetic isotope effect when 5′-^{3}H was the label for AMP with natural abundance isotope in the sensitive positions. The correction decreased the observed kinetic isotope effect when 5′-^{3}H was the label for AMP with heavy atom isotope in the sensitive positions.

Discussion

Isotope Effects on the Acid-Catalyzed Hydrolysis of AMP. Each heavy atom substitution of AMP gives information regarding the movement or the geometry of that atom in the transition state. The acid-catalyzed solvolysis of AMP provides a chemical model for the reaction catalyzed by AMP nucleosidase and demonstrates the information that can be obtained from each isotopic substitution. Chemical and previous heavy atom kinetic isotope effects on the acid-catalyzed solvolysis of adenosine, inosine, AMP, and related analogues have provided evidence that hydrolysis of the N-glycosidic bond follows protonation of N1 and N7 of the purine ring, leading to a transition state with considerable oxycarbonium[1] character in the ribose ring (Garrett & Mehta, 1972; Romero et al., 1978). These conclusions were originally based on the α-^{2}H secondary kinetic isotope effect for the acid-catalyzed solvolysis of inosine and adenosine and were strengthened and confirmed by the measurement of the primary ^{14}C and α-^{3}H secondary kinetic isotope effects with inosine and AMP (Parkin et al., 1984). α-Secondary kinetic isotope effects for hydrolysis of the N-glycosidic bond report primarily on the out-of-plane bending of H1′ in the transition state. Thus the α-^{2}H or α-^{3}H secondary isotope effects are relatively large for an S_N1-like transition state and small for an S_N2-like transition state. β-Secondary kinetic isotope effects for this reaction result from changes in vibrational frequency involving H2′ in the transition state. Since a major cause of this motion is the degree of hyperconjugation between C1′ and C2′ of the ribose ring, the β isotope effect should be relatively large for an S_N1-like transition state and small for an S_N2-like transition state with no carbocation character. Primary ^{14}C and ^{15}N isotope effects reflect the reaction coordinate motion of these atoms in the transition state and altered vibrational frequency due to decreased bond strengths. The largest primary ^{14}C kinetic isotope effect in glycoside hydrolysis of AMP would be expressed in a symmetric S_N2 transition state with movement of C1′ in the reaction coordinate from adenine toward the attacking nucleophile. This effect would be smaller for an S_N1 mechanism where the major motion in the reaction coordinate is separation of the N9 of departing adenine and the oxycarbonium of ribose 5-phosphate. Thus, the primary ^{15}N kinetic isotope effect would be largest for low C1′–N9 bond order in the transition state and smaller for a transition state where the C1′–N9 bond order is greater.

Table II: Approximate Ranges of Kinetic Isotope Effects Expected for S_N1 and S_N2 Hydrolysis of AMP

isotopic substitution in AMP	expected magnitude of kinetic isotope effect[a]	
	transition state with S_N1 character	transition state with S_N2 character
primary ^{14}C	1.00–1.05	1.09–1.14
primary ^{15}N	1.02–1.04[b]	1.01–1.02
α-secondary ^{2}H	1.10–1.40	1.00–1.10
β-secondary ^{2}H	1.05–1.15	1.00–1.02

[a] Kinetic isotope effects are expressed as k_{normal}/k_{heavy}, where k is the rate constant for acid-catalyzed hydrolysis or V/K for enzyme-catalyzed hydrolysis. Discussions of the magnitude of specific kinetic isotope effects for reactions with S_N1- and S_N2-like transition states can be found in Melander and Saunders (1980) and Cleland et al. (1977). The values can also be calculated by the methods described in the following paper (Mentch et al., 1987). Values in the table are a combination of calculated and literature values. [b] Calculated ^{15}N isotope effects can be as large as 1.04; however, chemical studies rarely result in effects greater than 1.03.

A summary of these isotope effects and the expected magnitude of each is given in Table II for mechanisms with S_N1 and S_N2 character. Comparison of these values with kinetic isotope effects for the acid-catalyzed solvolysis of AMP clearly indicates an S_N1-like transition state. The α-^{3}H secondary and primary ^{14}C effects were discussed previously (Parkin & Schramm, 1984) and indicate considerable sp^2 hybridization of C1′ in the transition state. The primary ^{14}C isotope effect of 1.044 reflects a significant motion of carbon in the transition state. The significant ^{14}C effect suggests that the carboxonium is incompletely formed in the transition state, since the expected ^{14}C isotope effect is nearer 1.00 for an S_N1-like transition state with a high degree of C–N bond cleavage and with a completely developed charge on the ribose ring (Mentch et al., 1987; Sinnott, 1986). The β-^{2}H secondary isotope effect of 1.077 ± 0.002 for acid-catalyzed hydrolysis of AMP is in agreement with the value of 1.073 ± 0.003 reported for the hydrolysis of methyl α-glucopyranoside, a reaction that is thought to have a transition state with a fully developed carboxonium ion (Sinnott, 1986).

The values of both α-^{2}H secondary and α-^{3}H secondary isotope effects for acid-catalyzed hydrolysis of AMP permit calculation of the Swain relationship (Swain et al., 1958), which is expected to be (α-^{2}H secondary isotope effect)$^{1.44}$ = (α-^{3}H secondary isotope effect). Although this relationship is difficult to determine accurately with small kinetic isotope effects (Northrop, 1982), the data for acid hydrolysis of AMP give a Swain exponent of 1.66, in reasonable agreement considering the inaccuracy that can arise from error in measuring the isotope effects, the extent of ^{2}H substitution in [1′-^{2}H,5′-^{3}H]AMP, and the variation from 1.44 that may occur in the Swain relationship. The α-secondary ^{3}H kinetic isotope effect is not subject to these uncertainties and is likely to be the more accurate of the values.

Combined kinetic isotope effects (multiple heavy atom substitutions in the same molecule) are expected to equal the product of the individual isotope effects in reaction mechanisms with a single transition state that expresses intrinsic isotope effects. The combined isotope effects give good agreement for expected and measured multiple isotope effects in the acid-catalyzed solvolysis of AMP (Table III). A combined ^{14}C and ^{15}N heavy atom kinetic isotope effect has not been previously reported to our knowledge. The combined isotope effect increases the magnitude of the heavy atom effects,

[1] The terms oxycarbonium and carboxonium refer to the developing cationic nature of C1′–O4′ of ribose in a S_N1-like transition state. Although it has been proposed that "carbonium" be reserved for pentacoordinated positive ions and "carbenium ion" be used for carbon cation centers (Olah, 1972), the term "oxycarbenium" is not in general use, hence the use of the above-mentioned terms.

Table III: Combined Kinetic Isotope Effects for Acid and Enzymatic Hydrolysis of AMP

combined kinetic isotope effect	acid catalyzed		enzyme catalyzed, no MgATP		enzyme catalyzed, plus ATP	
	expected[a]	obsd	expected	obsd	expected	obsd
primary ^{14}C and α-secondary ^{2}H	1.172 ± 0.005	1.158 ± 0.004	1.082 ± 0.003	1.084 ± 0.004	1.063 ± 0.004	1.058 ± 0.002
primary ^{14}C and primary ^{15}N	1.075 ± 0.004	1.064 ± 0.003	1.066 ± 0.004	1.064 ± 0.002	1.058 ± 0.003	1.048 ± 0.003

[a] Expected kinetic isotope effects were calculated as the product of the individual isotope effects taken from Table I.

providing increased sensitivity for the relatively small individual isotope effects.

In summary, the isotope effects for acid-catalyzed hydrolysis of AMP indicate that the transition state for the acid-catalyzed *N*-glycoside hydrolysis of AMP has considerable carboxonium character, with the C–N bond breaking well-developed and significant hyperconjugation between C1′ and C2′ of the ribose. The participation of a water nucleophile in carboxonium ion reactions has been discussed by Jencks (1980), and the isotope effects for AMP hydrolysis can accommodate a weak participation (or preassociation) by a water nucleophile which can contribute to the significant primary ^{14}C isotope effect (Mentch et al., 1987).

Isotope Effects on the Enzyme-Catalyzed Hydrolysis of AMP. Kinetic isotope effects for enzymatic reactions can be obscured by substrate addition that is not readily reversible (the so-called "sticky substrate"; Cleland, 1979) or by multiple steps in the transition state [e.g., Northrop (1981)]. Studies on the mechanism of AMP nucleosidase have indicated that AMP has no significant commitment to catalysis and that catalysis is rate limiting even when the chemical step is rapid, i.e., in the presence of the allosteric activator (DeWolf et al., 1986). The reaction catalyzed by AMP nucleosidase appears to consist of a discrete transition state since there is no evidence for a mechanism that contains a covalent enzyme–ribose 5-phosphate intermediate (DeWolf et al., 1986).

Isotope effects that are obscured (by a step following the isotopically sensitive step) would be diminished to the same degree if allosteric activator acted by decreasing the energy of activation of the rate-limiting step. The α-secondary ^{2}H and α-secondary ^{3}H isotope effects decreased by factors of 1.50 ± 0.14 and 1.47 ± 0.09, respectively, as the reaction rate increased approximately 200-fold due to the presence of the allosteric activator. In contrast, the heavy atom primary ^{14}C and primary ^{15}N isotope effects decreased by factors of 1.09 ± 0.09 and 1.20 ± 0.16, respectively. Thus the isotope effects are not equally sensitive to allosteric activation. This pattern indicates that the isotope effects observed with allosteric activation are intrinsic and are not obscured by a common slow step in the mechanism.

Intrinsic isotope effects are also implicated when observed isotope effects are near the theoretical maximum for a given transition-state structure. For AMP nucleosidase three kinetic isotope effects, namely, those of the β-secondary ^{2}H, the primary ^{14}C, and the primary ^{15}N isotope effects, are near the values observed in the acid hydrolysis of AMP. In the absence of allosteric activator, the β-secondary ^{2}H isotope effect of 1.067 ± 0.002 is near the experimental value of 1.073 ± 0.003 reported for a fully developed oxycarbonium transition state in the hydrolysis of methyl α-glucopyranoside (Sinnott, 1986) and is close to the value of 1.077 ± 0.002 for the acid-catalyzed solvolysis of [2′-^{2}H]AMP. This experiment also eliminates the transition states most likely to occur in a covalent mechanism with two nucleophilic displacements at C1′ of ribose. The β-secondary ^{2}H isotope effect would be near 1.00 for an S_N2-like transition state. Any other mechanism with two kinetically significant steps would also tend to obscure the magnitude of this effect. The primary ^{15}N kinetic isotope effects also indicate the intrinsic nature of the kinetic isotope effects since there is no significant difference in the acid- and enzyme- (no ATP) catalyzed kinetic isotope effects (Table III). The ^{15}N isotope effect of 1.030 approaches the theoretical limit of 1.040 for ^{15}N isotope effects. This result establishes that the modest α-secondary isotope effects reflect transition-state structure rather than suppressed isotope effects. It is also significant that the primary ^{14}C kinetic isotope effect is essentially unchanged by the presence of MgATP.

In the presence of ATP, where the catalytic rate is greatest, the kinetic isotope effect for conversion of [1-^{3}H]ribose 5-phosphate and adenine to AMP by the reverse reaction of AMP nucleosidase is approximately equal to the isotope effect with [1′-^{3}H]AMP in the forward reaction (Table I). The chemistry of N-glycosidic bond hydrolysis of AMP predicts a $H/^{3}H$ K_{eq} isotope effect near 1.00;[2] therefore, the ^{3}H V/K isotope effects for the forward and reverse reactions should be equal for the case where intrinsic isotope effects are expressed and a single isotopically sensitive step is present in the transition state. This result together with the isotope effects discussed above indicates that AMP nucleosidase expresses intrinsic V/K kinetic isotope effects.

Enzymatic hydrolysis of the N-glycosidic bond of AMP is most likely to occur by one of two mechanisms. The first is a mechanism reminiscent of acid-catalyzed solvolysis in which the enzyme would protonate or hydrogen bond the adenine ring to make a better leaving group, activate the incipient water nucleophile, and strain the glycosidic bond through the use of binding energy (DeWolf et al., 1979). The alternative double-displacement reaction in which a covalent enzyme–ribose 5-phosphate intermediate is present is eliminated by the magnitude of the β-secondary ^{2}H isotope effect. In addition to the kinetic and chemical studies that support a concerted mechanism (DeWolf et al., 1986), the multiple isotope effects can be used to distinguish single and multiple transition states (Melander & Saunders, 1980). Cleland's laboratory has extended these procedures to the case of multiple intermediates in enzymatic reactions (Hermes et al., 1982; Scharschmidt et al., 1984). If two heavy atom substitutions are present that express isotope effects in two sequential, isotopically sensitive steps in the transition state, the presence of one isotope effect will partially obscure the second. If a single isotopically sensitive transition state exists, the presence of multiple heavy atom substitutions will cause an increased isotope effect that is the product of the individual isotope effects. As summarized in Table III, multiple isotope effects for acid- and enzyme-catalyzed hydrolysis of AMP follow the same relationship, supporting the proposal that isotope effects are intrinsic for AMP nucleosidase and arise from a single rate-determining step in the reaction mechanism. Under these conditions, alterations in observed kinetic isotope effects reflect alterations

[2] The equilibrium isotope effect for [1-^{3}H]ribose 5-phosphate ⇌ [1′-^{3}H]AMP (ratio of K_{eq} for the light isotope/K_{eq} for the heavy isotope) is expected to be near unity since ^{3}H remains attached to a tetrahedral carbon in both substrate and product and the masses of the replacing atoms (oxygen and nitrogen) are similar. A comprehensive discussion of equilibrium isotope effects with examples is given by Cleland (1980).

in the transition-state structure.

The transition state for AMP nucleosidase in the absence of MgATP resembles that in acid-catalyzed solvolysis in terms of the primary ^{14}C and ^{15}N kinetic isotope effects. The reaction coordinate motion contains a significant contribution from C1′ of ribose, and the C1′ to N9 glycosidic bond is nearly broken. Additional evidence for the S_N1 characteristic comes from the large β-secondary ^2H isotope effect, indicating that the positive charge on the ribosyl ring oxygen is almost completely developed in the transition state. However the α-secondary ^2H and α-secondary ^3H effects are strongly suppressed in the enzyme-catalyzed reaction, indicating that out-of-plane bending of the 1′-H is hindered. Such suppression would occur if the enzyme enforces the nucleophilic participation of a water molecule as proposed from solvent participation studies (DeWolf et al., 1986) and if significant bond order remains in the N-glycosidic bond. These groups would restrict motion of 1′-H to give a kinetic isotope effect much smaller than that of the acid-catalyzed reaction where 1′-H motion is unrestricted in the carboxonium intermediate. This transition state would have many characteristics of an S_N1 transition state with the exception that the water molecule and departing adenine would have significant bond character to C1′ of the ribose. Specific models for acid- and enzyme-catalyzed transition states are given in the following paper (Mentch et al., 1987).

In the presence of MgATP, the isotope effects with α- and β-deuterium and -tritium decrease significantly. This effect can result from a compression of the transition-state structure, i.e., a transition state with higher bond orders to the departing adenine and/or the incoming water nucleophile. The incipient H_2O nucleophile or N9 of adenine has developed or retained increased bond order to C1′ of ribose while the reaction coordinate motion of C1′ remains largely unchanged. Although the structure becomes more associative in the presence of MgATP as evidenced by the reduced movement of H1′ (reduced α-primary effects) and reduced hyperconjugation to C2′ (reduced isotope effect on H2′), the S_N1 character predominates, since a primary ^{14}C isotope effect of >1.10 and a β-secondary ^2H effect near 1.00 would be expected for a symmetric S_N2-like transition state. The availability of intrinsic heavy atom isotope effects for both of the primary atoms in the reaction, two α-secondary effects, and one β-secondary effect permits calculations to establish the transition-state structures for these reactions. These techniques and the quantitative determination of the transition-state structure for the acid and enzymatic hydrolysis of AMP are given in the following paper (Mentch et al., 1987).

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Registry No. AMP, 61-19-8; [1′-^{2}H,5′-^{14}C]AMP, 106114-90-3; [1′-^{2}H,1′-^{14}C]AMP, 106114-91-4; [1′-^{2}H,5′-^{3}H]AMP, 106114-92-5; NADP, 53-59-8; [1′-^{2}H,5′-^{14}C]ATP, 106114-96-9; [1′-^{2}H,1′-^{14}C]ATP, 106114-97-0; [1′-^{2}H,5′-^{3}H]ATP, 106114-98-1; [2′-^{2}H,5′-^{3}H]AMP, 106114-99-2; [2′-^{2}H,5′-^{3}H]ATP, 106115-00-8; [9-^{15}N,5′-^{3}H]AMP, 106115-01-9; [9-^{15}N,1′-^{14}C]AMP, 106115-02-0; $^{15}NH_3$, 34819-78-8; AMP nucleosidase, 9025-45-0; [2-^{14}C]glucose, 3646-71-7; [6-^{14}C]-glucose, 3573-62-4; [6-^{3}H]glucose, 3615-68-7; [2-^{14}C]glucose 6-≤phosphate, 106114-93-6; [6-^{14}C]glucose 6-phosphate, 106114-94-7; [6-^{3}H]glucose 6-phosphate, 106114-95-8; [9-^{15}N]adenine, 56777-22-1; 5-amino-4,6-dichloropyrimidine, 5413-85-4; 4-chloro-5,6-diamino-pyrimidine, 4316-98-7; 6-chloropurine, 87-42-3; diethoxymethyl acetate, 14036-06-7.

REFERENCES

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104–125.
Cleland, W. W. (1982) *Methods Enzymol. 87*, 625–641.
Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds. (1977) *Isotope Effects on Enzyme-Catalyzed Reactions*, pp 100–120 and 233–251, University Park Press, Baltimore.
DeWolf, W. E., Jr., Fullin, F. A., & Schramm, V. L. (1979) *J. Biol. Chem. 254*, 10868–10875.
DeWolf, W. E., Jr., Markham, G. D., & Schramm, V. L. (1980) *J. Biol. Chem. 255*, 8210–8215.
DeWolf, W. E., Jr., Emig, F. A., & Schramm, V. L. (1986) *Biochemistry 25*, 4132–4140.
Fowler, A. V., & Smith, P. J. (1983) *J. Biol. Chem. 258*, 10204–10207.
Garrett, E. R., & Mehta, P. J. (1972) *J. Am. Chem. Soc. 94*, 8532–8541.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106–5114.
Hochstadt, J. (1978) *Methods Enzymol. 51*, 558–567.
Jencks, W. P. (1980) *Acc. Chem. Res. 13*, 161–169.
Legler, G., & Herrchen, M. (1984) *Eur. J. Biochem. 138*, 527–531.
Melander, L., & Saunders, W. H., Jr. (1980) in *Reaction Rates of Isotopic Molecules*, pp 276–280 and Chapter 6, Wiley, New York.
Mentch, F., Parkin, D. W., & Schramm, V. L. (1987) *Biochemistry* (following paper in this issue).
Northrop, D. B. (1981) *Annu. Rev. Biochem. 50*, 103–131.
Northrop, D. B. (1982) *Methods Enzymol. 87*, 607–640.
Olah, G. A. (1972) *J. Am. Chem. Soc. 94*, 808–820.
Parkin, D. W., & Schramm, V. L. (1984) *J. Biol. Chem. 259*, 9418–9425.
Parkin, D. W., Leung, H. B., & Schramm, V. L. (1984) *J. Biol. Chem. 259*, 9411–9417.
Romero, R., Stein, R., Bull, H. G., & Cordes, E. H. (1978) *J. Am. Chem. Soc. 100*, 7620–7624.
Scharschmidt, M., Fisher, M. A., & Cleland, W. W. (1984) *Biochemistry 23*, 5471–5478.
Schramm, V. L. (1974) *J. Biol. Chem. 249*, 1729–1736.
Sethi, S. K., Gupta, S. P., Jenkins, E. E., Whitehead, C. W., Townsend, L. B., & McCloskey, J. A. (1982) *J. Am. Chem. Soc. 104*, 3349–3353.
Sinnott, M. L. (1986) in *Mechanism of Enzymatic Reactions: Stereochemistry* (Frey, P. A., Ed.) pp 293–305, Elsevier, New York.