AMP Nucleosidase: Kinetic Mechanism and Thermodynamics†

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Received December 20, 1985; Revised Manuscript Received March 12, 1986

ABSTRACT: The kinetic mechanism of AMP nucleosidase (EC 3.2.2.4; AMP + H_2O → adenine + ribose 5-phosphate) from *Azotobacter vinelandii* is rapid-equilibrium random by initial rate studies of the forward and reverse reactions in the presence of MgATP, the allosteric activator. Inactivation-protection studies have established the binding of adenine to AMP nucleosidase in the absence of ribose 5-phosphate. Product inhibition by adenine suggests a dead-end complex of enzyme, AMP, and adenine. Methanol does not act as a nucleophile to replace H_2O in the reaction, and products do not exchange into substrate during AMP hydrolysis. Thus, the reactive complex has the properties of concerted hydrolysis by an enzyme-directed water molecule rather than by formation of a covalent intermediate with ribose 5-phosphate. The V_{max} in the forward reaction (AMP hydrolysis) is 300-fold greater than that in the reverse reaction. The K_{eq} for AMP hydrolysis has been experimentally determined to be 170 M and is in reasonable agreement with K_{eq} values of 77 and 36 M calculated from Haldane relationships. The equilibrium for enzyme-bound substrate and products strongly favors the enzyme–product ternary complex ([enzyme–adenine ribose 5-phosphate]/[enzyme–AMP] = 480). The temperature dependence of the kinetic constants gave Arrhenius plots with a distinct break between 20 and 25 °C. Above 25 °C, AMP binding demonstrates a strong entropic effect consistent with increased order in the Michaelis complex. Below 20 °C, binding is tighter and the entropic component is lost, indicating distinct enzyme conformations above and below 25 °C. Similar effects are seen on the energy of activation, which has $\Delta H^{\ddagger}$ of 11.4 kcal/mol at 30 °C and 19.1 kcal/mol at 10 °C. These thermodynamic parameters have been used to construct a reaction coordinate diagram for the enzyme.

AMP nucleosidase (EC 3.2.2.4) is an example of the nucleoside(tide) glycohydrolases in which a C–N glycosidic bond is hydrolyzed. This class of enzymes also includes NAD glycohydrolases, nucleoside glycohydrolases, and the purine and pyrimidine glycohydrolases for polynucleotide base excision. The kinetic mechanisms for NAD glycohydrolases from calf spleen and bull semen have been proposed on the basis of product inhibition and chemical and isotope exchange studies (Schuber et al., 1976; Yuan & Anderson, 1973) and involve a sequential release of products with the ADP-ribosyl moiety being released last. In addition, it has been postulated that these enzymes undergo formation of a covalent enzyme–ADP-ribose intermediate. The hydrolysis of the covalent intermediate is at least partially rate limiting, since many of these enzymes can effect a relatively rapid transglycosylation (base exchange) reaction during the initial rate period of substrate hydrolysis. Secondary deuterium isotope effects are consistent with covalent catalysis for NAD glycohydrolase. Isotope effect studies with purine nucleoside phosphorylase suggest that glycosidic bond cleavage becomes rate limiting[1] with respect to V/K parameters for these enzymes only when pH values cause a decrease in catalytic rate (Stein & Cordes, 1981).

The purpose of this study was to determine the kinetic mechanism and thermodynamic properties of the allosteric AMP nucleosidase from *Azotobacter vinelandii*. Studies of the substrate specificity of this enzyme have suggested that N-glycosidic bond cleavage is a rate-determining step of the reaction and that strain of the glycosidic bond may play a significant role in bond breaking (DeWolf et al., 1979). These suggestions have been strengthened by heavy-atom isotope effects with AMP nucleosidase, which indicate that substrate has a low commitment to catalysis (Parkin et al., 1984; Parkin & Schramm, 1984).

However, these studies were V/K effects, which cannot identify rate-limiting steps that occur beyond the release of the first product. In this study, the kinetic mechanism has been investigated for both the forward and reverse reactions of AMP nucleosidase in the presence of near-saturating MgATP, the allosteric activator. The energetics of the overall reaction and of many of the individual steps have been established by identification of the kinetic mechanism and assignment of the individual kinetic constants to discrete steps in the enzyme-catalyzed reaction. This information can be used in conjuction with multiple kinetic isotope effects to provide comprehensive information on the nature of the transition state for the reaction catalyzed by AMP nucleosidase.

Experimental Procedures

Materials. The disodium salt of ribose 5-phosphate was obtained from Calbiochem and [^{14}C]methanol was purchased from Amersham. The dipotassium salt of ribose 5-phosphate was obtained by passing the sodium salt through a Dowex-50 (H^+ form) column and titrating the resulting solution to pH 7 with KOH. Labeled methanol was used as provided or was further purified by distillation with a liquid nitrogen trap. Reagent-grade methanol was a product of Mallinckrodt. AMP

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[1] The term "rate-limiting step" refers to the step that has the highest energy of activation on the reaction coordinate. It is recognized that many reactions will not have a single step that is rate-limiting and that during steady-state hydrolysis the net forward flux is the same through each step of the reaction. The terms "rate limiting" and "rate determining" are meant to have the same meaning.

0006-2960/86/0425-4132$01.50/0

nucleosidase was purified from *Azotobacter vinelandii* as previously described (Schramm & Leung, 1978). AMP deaminase was purchased from Sigma or was prepared from rat muscle (Coffee, 1978). Synthesis of methyl riboside 5-phosphate was by the method of Switzer and Simcox (1974). Purity of the product was confirmed by inorganic phosphate, total phosphate, and reducing sugar content before and after acid hydrolysis.

Product Inhibition with Adenine. Initial reaction rates were determined by measuring the production of reducing sugar at 30 °C in reaction mixtures containing 0.1 M triethanolamine hydrochloride, pH 8.0, 1 mM $MgCl_2$, 0.5 mM ATP, and AMP in concentrations varying from 0.05 to 1 mM. The concentration of adenine was varied over the range 0–2 mM. In experiments using a high concentration of MgATP, the reaction mixtures contained 10 mM $MgCl_2$ and ATP. The concentrations of $MgCl_2$ were chosen to provide essentially complete conversion of ATP to MgATP and to give noninhibitory concentrations of free Mg^{2+}.

Product Inhibition with Ribose 5-Phosphate. Initial reaction rates were determined by the rate of [8-^{14}C]adenine production from [8-^{14}C]AMP at 30 °C in the presence of varying concentrations of ribose 5-phosphate and unlabeled adenine, by the method described previously (Schramm & Reed, 1980). The incubation mixtures for ribose 5-phosphate concentrations of 30 mM or below contained 0.1 M triethanolamine hydrochloride, pH 8.0, 2 mM $MgCl_2$, 1 mM ATP, and [8-^{14}C]AMP fixed at 0.16 mM. The concentration of unlabeled adenine was varied from 0 to 2 mM, and ribose 5-phosphate was varied from 0 to 30 mM. Varying amounts of NaCl were added to maintain the concentration of Na^+ at a constant 60 mM at all concentrations of ribose 5-phosphate. Incubation mixtures for ribose 5-phosphate concentrations of 100–300 mM contained 0.7 M triethanolamine adjusted to pH 8.0 with HCl or the acid form of ribose 5-phosphate. Appropriate mixtures of triethanolamine hydrochloride and triethanolamine–ribose 5-phosphate were used to provide the desired concentration of ribose 5-phosphate. MgATP was maintained at 2 mM, and free magnesium ion was fixed at 0.1 mM with 10 and 0.1 mM for the dissociation constants of the Mg–ribose 5-phosphate and MgATP complexes, respectively, at these relatively high ionic strengths (O'Sullivan & Smithers, 1979).

Effect of Temperature on V_{max} and K_m. The temperature coefficient of 0.1 M triethanolamine (pH 8.0) was determined over the temperature range 5–45 °C in incubation mixtures containing 1 mM $MgCl_2$, 0.5 mM ATP, and 0.2 mM AMP. Reaction mixtures for initial rate measurements contained the same ingredients to give pH 8.0 and AMP at concentrations varying from 0.05 to 1 mM. The reaction mixtures were equilibrated at least 10 min at the desired temperature before addition of the enzyme. Initial reaction rates were measured by the rate of production of reducing sugar.

Determination of Equilibrium Constant. The K_{eq} for the conversion of adenine and ribose 5-phosphate to AMP was estimated in reaction mixtures that were near equilibrium. Reaction mixtures of 10–22 μL contained 0.1 M triethanolamine hydrochloride, pH 8.0, 1–2 M ribose 5-phosphate (potassium salt), 20 mM adenine, 24 mM $MgCl_2$, and 20 mM sodium tripolyphosphate. Tripolyphosphate rather than ATP was used as the allosteric activator to prevent traces of AMP from being introduced with ATP. Use of tripolyphosphate also eliminates the possibility that adenylate kinase, which might be present as a trace contaminent, would act on AMP formed by the reverse reaction of AMP nucleosidase. Previous investigation has demonstrated the activation of AMP nucleosidase by magnesium pyrophosphate (Schramm, 1974), and magnesium tripolyphosphate is slightly more effective as the allosteric activator. Reactions were initiated with 0.1–0.2 unit of AMP nucleosidase, and samples of 3 μL were taken at appropriate time intervals. Samples were quenched in 20 μL of 0.05 M HCl, incubated for 30 min, and neutralized with 20 μL of 0.05 M NaOH. The concentration of AMP and adenine was determined by relative peak area following chromatography of samples on a Waters C-18 μBondapak HPLC column eluted with 0.1 M ammonium acetate, pH 5.0. Standards with known amounts of AMP, adenine, enzyme, and reaction mixture were also analyzed to quantitate the system. Analysis of multiple samples with ratios of adenine/AMP similar to those in actual experiments gave standard deviations of less than 10% of the known values.

Reverse Reaction of AMP Nucleosidase. The formation of AMP from adenine and ribose 5-phosphate was measured by coupling the reaction to the reactions catalyzed by adenylate kinase and pyruvate kinase. AMP deaminase was tested as a coupling system and was unsatisfactory even at several milligrams per milliliter enzyme due to the unfavorable equilibrium of the AMP nucleosidase reaction (see Results) and unfavorable V_{max}/K_m of AMP deaminase for AMP (Coffee, 1978).

Reaction mixtures for the reverse reaction of AMP nucleosidase contained 0.1 M triethanolamine hydrochloride, pH 8.0, 3 mM ATP, 1 mM phosphoenolpyruvate, 0.2 M potassium ion, and 0.2 μCi of [8-^{14}C]adenine, the desired concentrations of adenine and ribose 5-phosphate (potassium salt), 250 units/mL adenylate kinase, and 250 units/mL pyruvate kinase in a final volume of 30 μL. Reactions were initiated by the addition of 0.03–0.1 unit/mL AMP nucleosidase. At appropriate intervals, samples were removed and spotted onto Whatman 3MM filter paper previously spotted with 10 μL of 100 mM ethylenediaminetetraacetic acid (EDTA) containing 10 mM adenine, 10 mM AMP, 10 mM ATP, and 10 μM formycin 5′-phosphate, a tight binding inhibitor of AMP nucleosidase (DeWolf et al., 1979). The chromatograms were developed in the aqueous phase of a 1:1 mixture of 8.3% (w/v) $Na_2HPO_4 \cdot 7H_2O$ in water–isoamyl alcohol for approximately 4 h. The chromatograms were dried, the adenine and ATP spots were excised, and the radioactivity was determined by scintillation counting. In control experiments, the AMP spots were also excised and were confirmed as containing none of the radioactivity, thus demonstrating the efficiency of coupling from adenine to ATP. At least two time periods were analyzed from each reaction mixture to ensure that initial rates were being measured. The free magnesium concentration was maintained at 0.1 mM by assuming that the dissociation constants are 10 mM for magnesium ribose 5-phosphate and 100 μM for MgATP in the presence of these relatively high ionic strength solutions (O'Sullivan & Smithers, 1979).

Exchange of Products into AMP. AMP nucleosidase was used to prepare [U-^{14}C]ribose 5-phosphate from [U-^{14}C]AMP. Labeled ribose 5-phosphate was purified from other components of the reaction mixture by chromatography on G-10 Sephadex. Reaction mixtures for the exchange of [U-^{14}C]-ribose 5-phosphate into AMP during the initial rate of AMP hydrolysis contained 50 mM triethanolamine hydrochloride, pH 8.0, 300 μM ATP, 1 mM $MgCl_2$, 2 mM AMP, and 5 mM [U-^{14}C]ribose 5-phosphate, 8.8 μCi/μmol, in a total volume of 33 μL. Samples (1 μL) were placed on Whatman 3MM chromatography paper previously spotted with 6 μL of 100 mM EDTA containing 10 mM AMP and 10 mM adenine and

9 μL of 10 μM formycin 5′-phosphate. Samples were taken at timed intervals after the addition of 9×10^{-3} unit of AMP nucleosidase. AMP and ribose 5-phosphate were separated by chromatography in 1 M ammonium acetate, pH 7.6. The AMP and ribose 5-phosphate spots were cut out and analyzed by scintillation counting. Similar experiments were performed in the presence of adenine and ribose 5-phosphate as described under Results. Reaction mixtures for the exchange of [8-^{14}C]adenine into AMP contained 1 mM [8-^{14}C]adenine (56 μCi/μmol). The concentrations of buffer, ATP, $MgCl_2$, and AMP were the same as in experiments described above for incorporation of ribose 5-phosphate into AMP. Control experiments contained [8-^{14}C]AMP as substrate and unlabeled adenine and ribose 5-phosphate as products. Samples were analyzed by chromatography as described above to ensure that the enzyme was actively converting AMP to products under the conditions of the experiments.

Incorporation of [^{14}C]Methanol into Ribose 5-Phosphate. The stability of AMP nucleosidase in buffers containing methanol was investigated with 1-mL incubation mixtures containing 0.1 M triethanolamine hydrochloride, pH 8.0, 2 mM $MgCl_2$, 2 mM ATP, and methanol in varying concentrations up to 40% by volume. At various time intervals after addition of enzyme, 40-μL aliquots were removed and assayed in a reaction mixture containing 0.1 M triethanolamine hydrochloride, pH 8.0, 0.5 mM ATP, 1 mM $MgCl_2$, and 4 mM AMP. The catalytic activity of AMP nucleosidase in reaction mixtures containing methanol was investigated with [8-^{14}C]-AMP as substrate similar to the assays described above. Reaction mixtures (50 μL) contained 0.1 M triethanolamine hydrochloride, pH 8.0, 2 mM $MgCl_2$, 1 mM ATP, 4 mM [8-^{14}C]AMP (0.4 μCi), and various concentrations of methanol up to 20%. Following the addition of enzyme and incubation at 30 °C, aliquots (5 μL) were removed at appropriate times and applied to Whatman 3MM paper. The chromatograms were developed in a solvent system consisting of 1-butanol–water–concentrated NH_4OH (5:1:0.05 by volume). The spots corresponding to AMP and adenine were cut out, and the radioactivity was determined by scintillation counting.

The enzymatic incorporation of [^{14}C]methanol as a reactant in the reaction catalyzed by AMP nucleosidase (to form 1-methylriboside 5-phosphate) was performed in a closed vacuum distillation apparatus. The incubation mixture of 1 mL contained 0.1 M triethanolamine hydrochloride, pH 8.0, 2 mM $MgCl_2$, 1 mM ATP, 20% by volume [^{14}C]methanol (25 μCi), and 4 mM AMP. Control incubation mixtures contained no AMP. The reaction was started by the addition of 0.2 unit of AMP nucleosidase and was continued for 30 min at 30 °C. This time was sufficient to obtain near equilibrium on the basis of the analysis of samples taken during the incubation. After the incubation, the solvent was replaced 5 times with 1 mL of unlabeled 20% methanol, and the sample was dissolved in 1 mL of H_2O. The reconstituted aqueous samples were applied to a column of Sephadex G-10 (0.9 × 100 cm) and eluted with 20 mM acetic acid. Fractions were analyzed for radioactivity, reducing sugar, adenine, and adenine nucleotides.

Analysis of Kinetic Data. Steady-state kinetic data were analyzed with the programs described by Cleland (1979). Product inhibition data and bisubstrate reaction data were analyzed as individual lines by computer fitting to the equation for a rectangular hyperbola. The slopes and intercepts of double-reciprocal plots of these lines were examined to determine if significant slope or intercept variations resulted from varied product or second substrate. After establishment of the significance of slope or intercept values, the data were fitted to the appropriate equation for competitive, noncompetitive, or sequential reaction mechanisms. Product inhibition data was commonly fitted to the equations for both competitive and noncompetitive inhibition.

Inactivation and Protection Studies. Initial rates of inactivation of AMP nucleosidase were patterned after the studies of Schramm and Fullin (1978). Enzyme was diluted to 1 μg/mL in 50 mM triethanolamine hydrochloride, pH 8.0, containing 1 mM potassium phosphate and varying concentrations of adenine. Samples were withdrawn at timed intervals from 15 s to 4 min and assayed for residual activity. The slope of activity remaining as a function of time was used as the initial rate of activity loss.

Results

Product Inhibition. The product inhibition pattern for adenine gave mixed-type inhibition with small ordinate intercept effects when AMP was the substrate and 0.5 mM MgATP was present as the allosteric activator. The best fit of these data to the equation for noncompetitive inhibition (Cleland, 1979) gave a K_{is} (slope inhibition constant) of 0.60 ± 0.04 mM and a K_{ii} (intercept inhibition constant) of 5.2 ± 0.7 mM compared to a K_m of 0.104 ± 0.005 mM for AMP. Although the best fit lines intersected close to the vertical axis, the inhibition pattern was clearly noncompetitive, since the intercept inhibition constant is significant. A fit of the same data to the equation for competitive inhibition resulted in the lines deviating from the experimental data. The results of one such experiment are given in Figure 1A.

Product inhibition by adenine was also examined in the presence of 5 mM MgATP to ensure that the allosteric activator was present at a near-saturating concentration. The results of the experiment are given in Figure 1B. Analysis of the experimental data indicated the best agreement with the equation for noncompetitive inhibition (Cleland, 1979) and gave a K_{is} of 0.53 ± 0.07 mM and a K_{ii} of 7.7 ± 1.8 mM. These values are not significantly different from those reported above for the lower MgATP concentration. The kinetic constants for adenine inhibition are summarized in Table I.

With the concentration of AMP fixed at 0.1 mM, the inhibition by 30 mM ribose 5-phosphate increased from 15% with no adenine to 50% at 2 mM adenine. The poor inhibition by ribose 5-phosphate and the combined inhibition by adenine and ribose 5-phosphate are illustrated in Figure 2. Product inhibition studies with higher concentrations of ribose 5-phosphate with respect to AMP gave linear competitive inhibition when AMP concentrations were varied from 100 to 500 μM and ribose 5-phosphate was present at 100, 200, and 300 mM. These relatively high product concentrations required constant ionic strength, which was provided by triethanolamine buffer as described under Experimental Procedures. Under these conditions, the K_i slope for ribose 5-phosphate was 110 ± 15 mM, and the K_m for AMP was 300 ± 56 μM. The use of ribose 5-phosphate as counterion to triethanolamine resulted in a change in chloride ion concentration from approximately 400 mM in the absence of ribose 5-phosphate to approximately 50 mM in the presence of 300 mM ribose 5-phosphate. The kinetic constants for the inhibition of AMP nucleosidase by ribose 5-phosphate are summarized in Table I.

Protection of Inactivation by Adenine. Addition of adenine to dilute solutions of AMP nucleosidase protected the enzyme from loss of activity (Figure 3). A plot of initial rate of activity loss as a function of adenine concentration gave an adenine concentration of 1.5 ± 0.4 mM for half-maximal protection from inactivation by dilution.

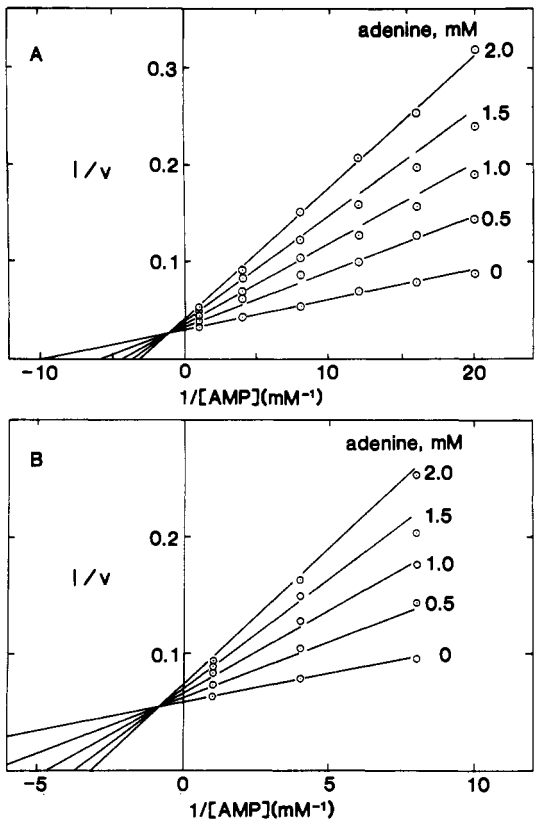


FIGURE 1: Inhibition of AMP nucleosidase by adenine. Initial rates were measured by the production of reducing sugar as described under Experimental Procedures. In panel A, $MgCl_2$ was fixed at 1.0 mM and the ATP was 0.5 mM. In panel B, reaction mixtures contained 10 mM $MgCl_2$ and 5 mM ATP. Under these conditions, nearly all of the ATP is bound to Mg^{2+} as the $MgATP^{2-}$ complex. The data points are the experimental rates, and the lines are drawn with the use of the constants obtained from a computer fit of the data to the equation for linear noncompetitive inhibition. Initial rates are expressed as μmol of ribose 5-phosphate formed min^{-1} (mg of protein)$^{-1}$.

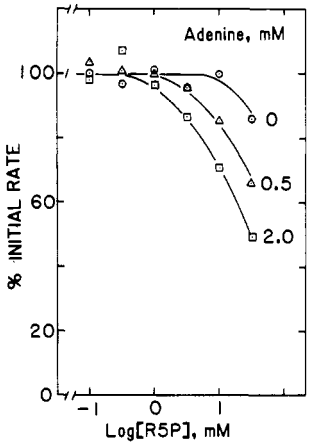


FIGURE 2: Inhibition of AMP nucleosidase by ribose 5-phosphate in the absence and presence of adenine. Initial rates of AMP hydrolysis were determined by measuring the production of [8-^{14}C]adenine from [8-^{14}C]AMP as described under Experimental Procedures. Total AMP concentration was 0.1 mM, and MgATP was 1 mM. The points represent experimental rates, and the lines were drawn by eye to the experimental points. Initial rate is expressed as percent of the initial rate measured in the absence of ribose 5-phosphate.

Equilibrium Constant. The K_{eq} for hydrolysis of AMP to adenine and ribose 5-phosphate was estimated by measuring the extent of conversion of ribose 5-phosphate and adenine to AMP. The equilibrium constant for AMP N-glycoside bond hydrolysis was therefore calculated by quantitating the relative concentrations of adenine and AMP by HPLC. The result of one such experiment is illustrated in Figure 4, and the results of additional experiments are summarized in Table II. With a variety of initial concentrations of ribose 5-phosphate, adenine, and enzyme, the calculated equilibrium constant, K_{eq} = [adenine][ribose 5-phosphate]/[AMP], averaged 277 M at concentrations of ribose 5-phosphate near 2 M. At ribose 5-phosphate concentrations of 1.4 and 0.9 M, the K_{eq} averaged 170 M.

Variations of V_{max} and K_m with Temperature. Plots of the variation of log V_{max}/T and log K_m with $1/T$ are given in Figure 5. A break in the log V_{max}/T plot occurs near 25 °C.

Table I: Kinetic Constants for AMP Nucleosidase[a]

kinetic constant	reaction	value ± SD	comment
V_1	AMP → Ad + R5P	34 ± 1 μmol min^{-1} mg^{-1}	*b*
V_2	Ad + R5P → AMP	0.11 ± 0.01 μmol min^{-1} mg^{-1}	*c*
K_{AMP}	E + AMP ⇌ E·AMP	0.104 ± 0.005 mM	*b*
$K_{is,R5P}$	E + R5P ⇌ E·R5P	110 ± 15 mM	*d*
$K_{ib,R5P}$	E + R5P ⇌ E·R5P	140 ± 60 mM	*c*
$K_{b,R5P}$	E·Ad + R5P ⇌ E·Ad·R5P	52 ± 4 mM	*c*
$K_{is,Ad}$	E + Ad ⇌ E·Ad	0.58 ± 0.03 mM	*b*
$K_{ii,Ad}$	E·AMP + Ad ⇌ Ad·E·AMP	6.7 ± 0.4 mM	*b*
$K_{a,Ad}$	E·R5P + Ad ⇌ E·R5P·Ad	0.10 ± 0.04 mM	*c*
$K_{ia,Ad}$	E + Ad ⇌ E·Ad	0.35 ± 0.05 mM	*c*

[a] For the rapid-equilibrium mechanism in Scheme I, kinetic constants represent rate or dissociation constants for the individual reactions. [b] Obtained from product inhibition by adenine with respect to AMP. Kinetic constants were obtained from the best fit of the experimental data to the equation for noncompetitive inhibition (Cleland, 1979). Kinetic constants are the weighted mean values from two experiments with 0.5 and 5 mM MgATP as allosteric activator as described under Results. [c] Obtained from the reverse reaction with adenine and ribose 5-phosphate as substrates. Kinetic constants were obtained from the best fit of the experimental data to the equation for a bisubstrate sequential kinetic mechanism (Cleland, 1979). Kinetic constants are the weighted mean values from three experiments. [d] Obtained from product inhibition with ribose 5-phosphate with respect to AMP. Kinetic constants were obtained from the best fit of the experimental data to the equation for competitive inhibition (Cleland, 1979). The kinetic constant is the weighted mean value of two experiments.

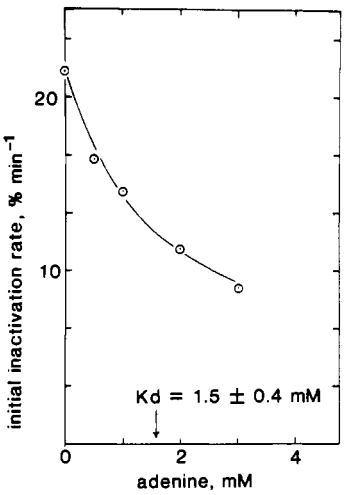


FIGURE 3: Effect of adenine on the rate of AMP nucleosidase inactivation. Loss of enzymatic activity was determined as a function of time after dilution of the enzyme into buffer, which caused the loss of 21% of the activity per minute. Each data point represents the initial rate of enzyme inactivation at the specified concentration of adenine. The line is the best fit of the data to the equation for a rectangular hyperbola.

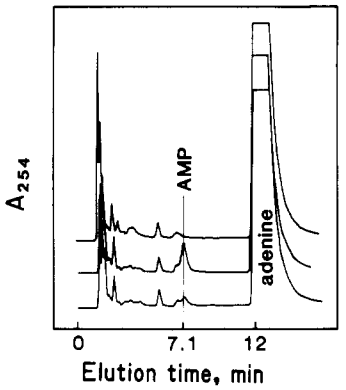


FIGURE 4: Determination of the K_{eq} for AMP nucleosidase by HPLC analysis of substrate and product ratio. Reaction mixtures containing 1.9 M ribose 5-phosphate (potassium salt) and 19 mM adenine were treated with HCl and neutralized with NaOH (see Experimental Procedures) before analysis by HPLC. The upper trace is the analysis of a reaction mixture to which no enzyme was added, but all other treatment was the same as other samples. The middle trace is a reaction mixture to which no enzyme was added but to which AMP had been added as an internal standard. The lower trace is an experiment in which enzyme was added to convert adenine and ribose 5-phosphate to AMP. The same settings were used for each analysis, and the ordinate absorbance settings are arbitrary but equivalent for the three samples. Relative area under each peak was estimated with a Hewlett-Packard integrating recorder.

Table II: Determination of Equilibrium Constant for Reaction Catalyzed by AMP Nucleosidase

reactants			AMP concn at equilibrium (μM)[a]	K_{eq} (M)[b]
adenine (mM)	ribose 5-phosphate (M)	enzyme (μM)		
19	1.9	2.1	137 ± 14	264 ± 26
18.2	1.82	4.2	114 ± 16	291 ± 40
18.2	1.36	1.1	141 ± 4	175 ± 5
18.2	0.91	1.1	101 ± 15	164 ± 25

[a] Average ± SD of AMP concentration determined in four individual samples taken after the reaction had reached equilibrium, as judged by no additional change in AMP concentration. [b] The equilibrium constant assumes the standard state of 1 M for H_2O; K_{eq} = [adenine][ribose 5-phosphate]/[AMP].

The standard errors of the V_{max} precluded the fitting of these data to a single straight line. Separate enthalpies may be calculated for each linear portion of the curve and are related to the slope by the expression: slope = $\Delta H^{\ddagger}/(2.3R)$. Above 25 °C, the calculated $\Delta H°$ for V_{max} is 10.8 kcal/mol and for temperatures below 25 °C is 18.5 kcal/mol.

The free energy change for the binding of substrate to enzyme may be calculated from the temperature dependence of K_m when K_m is a dissociation constant (see Discussion). Above 20 °C, the calculated $\Delta H°$ for the K_m is 12.7 kcal/mol. A break occurs in the temperature curve for the K_m near 20 °C, and at lower temperatures, a slope corresponding to a $\Delta H°$ of 6.4 kcal/mol is obtained. The free energy, enthalpy, and entropy values derived from the data of Figure 5 are summarized in Table III.

Hydrolysis of AMP in the Presence of Methanol. The stability and catalytic efficiency of AMP nucleosidase was determined at various concentrations of methanol as described under Experimental Procedures. At concentrations of methanol up to 20% by volume, AMP nucleosidase was stable for at least 1 h under conditions used for the kinetic studies. Above 20% methanol, the enzyme becomes increasingly unstable, and at 50% methanol, essentially all activity is lost after 1 min. The initial rates of AMP hydrolysis were determined in reaction mixtures containing 20% radioactive

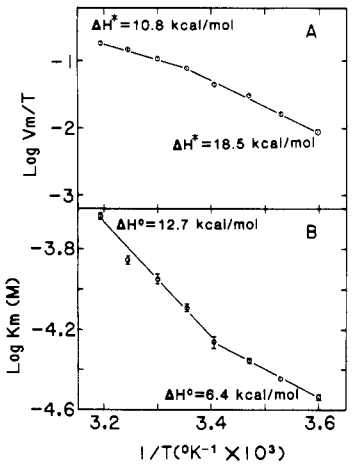


FIGURE 5: Variation of kinetic constants for AMP nucleosidase with temperature. Initial rates were measured at 5–40 °C with the reaction mixtures described under Experimental Procedures. The kinetic constants V_m and K_m were obtained from computer fits to the equation for a rectangular hyperbola (Cleland, 1979). Each point represents the weighted mean calculated from five experiments at each temperature. The equations for calculation of the weighted mean are given in Morrison and Uhr (1966). The lines were drawn by eye to fit the experimental points. Enthalpy of activation ($\Delta H^{\ddagger}$) in panel A is for the formation of the transition-state complex from the enzyme–AMP complex, which is near saturation with MgATP, the allosteric activator. The enthalpy for substrate–enzyme interaction ($\Delta H°$) in panel B is for the Michaelis constant, the dissociation constant for the enzyme–AMP complex. The standard deviations of the weighted mean values are given by standard error bars in (B). In (A), the errors lie within the dimensions of the data points.

Table III: Free Energy, Enthalpy, and Entropy Values for AMP Nucleosidase at 30 °C[a]

step	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (kcal mol^{-1} deg^{-1})
E + AMP ⇌ E + R5P + Ad	−2.8[a]	−3.1[b]	
E + AMP ⇌ E·AMP	−5.5	−12.7	−23.8
E·AMP ⇌ (E·R5P·Ad)‡	10.5[c]	10.8	1.1
E·AMP ⇌ E·R5P·Ad	−3.7[d]		
E·R5P·Ad ⇌ E·Ad + R5P	2.0		
E·Ad ⇌ E + Ad	4.4		

[a] Thermodynamic constants refer to the equilibrium constants that describe individual steps of the reaction. [b] Calculated from the average of the K_d values determined from the Haldane relationships and the experimentally determined values of 170 M. Water is taken to be at the standard state (1 M) in the K_{eq} value used for this calculation. The pH is 8.0. [c] The enthalpy for the overall reaction was estimated from values for the bond energies given in Long (1961) and is therefore only an approximation. [d] The correct designations for the thermodynamic parameters for this step are $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$, which represent the free energy, enthalpy, and entropy of activation, respectively. [e] Calculated from the thermodynamic equilibrium considerations in Scheme II. Water is assumed to be at the standard state of 1 M for this calculation.

methanol to establish whether methyl riboside 5-phosphate would be formed as product. The initial rate of AMP hydrolysis in the presence of 20% methanol was 68% of that in the absence of methanol.

Following the removal of methanol from the reaction mixture, the products were resolved on a Sephadex G-10 column. The column had been previously calibrated with methyl riboside 5-phosphate prepared according to the method of Switzer and Simcox (1974) and with ribose 5-phosphate. The peak corresponding to the elution position of methyl riboside 5-phosphate was analyzed for the incorporation of radioactivity from [^{14}C]methanol. The counts in the methyl riboside 5-phosphate peak corresponded to less than 0.01 μmol of methanol incorporation compared to the 4 μmol of ribose

5-phosphate produced. Thus, within the limits of detection, methanol did not participate as a nucleophile in the hydrolysis of AMP by AMP nucleosidase. The limit of [^{14}C]methanol incorporation into product was calculated by summing the counts contained in the peak position expected for methyl riboside 5-phosphate, compared to control samples that were incubated in the same way but without enzyme.

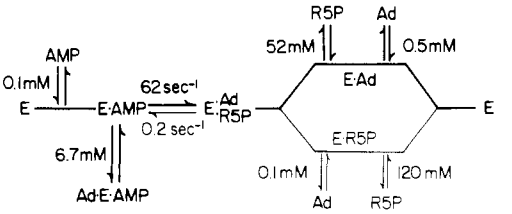
Kinetics of Reverse Reaction of AMP Nucleosidase. The equilibrium constant of 170 M for glycosidic bond hydrolysis of AMP suggested that it might be possible to observe the back-reaction provided that efficient removal of AMP could be accomplished. Coupling with adenylate kinase and pyruvate kinase proved satisfactory, permitting rapid conversion of AMP into the MgATP pool used for activation of AMP nucleosidase. Since the enzyme was already maximally activated, this small increase in the MgATP pool did not alter the rate of catalysis by AMP nucleosidase. The kinetics of the reverse reaction were obtained with [8-^{14}C]adenine and ribose 5-phosphate as substrates. When the reaction was run with dipotassium ribose 5-phosphate as variable substrate (varied from 20 to 100 mM), adenine as the fixed variable (0.2–5 mM), and 3 mM MgATP as allosteric activator, the kinetic pattern gave intersecting lines. Slope changes were more pronounced than ordinate intercept changes. Analysis of the experimental data gave good fits to the equation for a two-substrate sequential mechanism (Cleland, 1979). Kinetic constants obtained from three experiments gave the weighted mean values listed in Table I. The reverse reaction has a V_{max} only 0.003 that of the forward direction.

Exchange of Products into AMP. Incorporation of radioactive ribose 5-phosphate or adenine into AMP during the steady-state period of AMP hydrolysis was tested in the presence of one or both products. Under conditions of 2 mM AMP and 5 mM [U-^{14}C]ribose 5-phosphate, no incorporation of ^{14}C into AMP was observed under conditions that would have detected a ribose 5-phosphate exchange rate 6×10^{-3} that of the rate of AMP hydrolysis. Likewise, no incorporation of [U-^{14}C]ribose 5-phosphate into AMP was detected with 5 mM ribose 5-phosphate in the presence of 5 mM adenine under conditions that could have measured an incorporation rate 6×10^{-4} that of the rate of AMP hydrolysis. Under conditions of 2 mM AMP and 1 mM [8-^{14}C]adenine, no ^{14}C incorporation into AMP was observed under conditions that would have detected an adenine incorporation rate 7×10^{-4} that of the rate of AMP hydrolysis. No incorporation of adenine into AMP was observed with 1 mM [8-^{14}C]adenine and 20 mM ribose 5-phosphate under conditions that would have detected an adenine incorporation rate 7×10^{-4} that of the rate of AMP hydrolysis. For every experiment, controls established that AMP hydrolysis was occurring during the experiment. Samples were taken during the course of the reaction from <5% to >90% of AMP hydrolysis as described under Experimental Procedures. The initial rate period was approximately 20% of AMP hydrolysis under these experimental conditions.

Discussion

Kinetic Mechanism of AMP Nucleosidase. The competitive product inhibition pattern by ribose 5-phosphate and the noncompetitive pattern by adenine with respect to AMP is consistent with a simple ordered release of adenine followed by ribose 5-phosphate or a rapid-equilibrium random mechanism with a dead-end complex. Simple random (two noncompetitive patterns), rapid-equilibrium random (two competitive patterns), and rapid-equilibrium ordered (only one product inhibits and gives a competitive pattern) reaction mechanisms can be eliminated by these results. To distinguish

Scheme I: Kinetic Mechanism for Reaction Catalyzed by AMP Nucleosidase[a]



[a] Abbreviations are defined in the legend to Figure 6. The numbers represent dissociation constants for the release of individual reactants or the microscopic rate constants. The addition of water is not shown. The Ad·E·AMP complex is a dead-end complex.

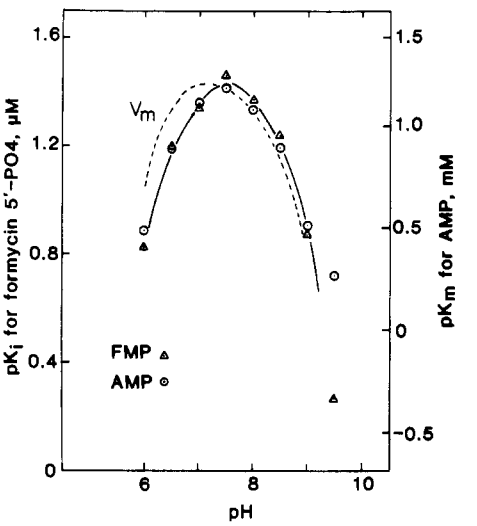


FIGURE 6: Effect of pH on kinetic constants for AMP nucleosidase. The methods and conditions for determination of the kinetic constants are given in DeWolf et al. (1979). The dashed line represents the effect of pH on the V_{max}. The solid line is drawn by eye to demonstrate the response of the K_m for AMP and the K_{is} for formycin 5'-phosphate to pH.

between the ordered mechanism and the rapid-equilibrium random mechanism with a dead-end complex, the interaction of adenine with free enzyme was investigated by inactivation studies. Adenine protected AMP nucleosidase against low ionic strength and phosphate-induced dissociation with an apparent protection constant of 1.5 mM (Figure 3). This constant is in good agreement with the kinetic constants of 0.58 and 0.35 mM for enzyme–adenine interactions, since phosphate and substrate are known to compete in the enzyme inactivation mechanism (Schramm & Fullin, 1978). The demonstration of direct adenine–enzyme and ribose 5-phosphate–enzyme interactions (competitive inhibition) is consistent with a rapid-equilibrium random reaction mechanism as indicated in Scheme I. Additional evidence for AMP being in equilibrium with the enzyme (low commitment to catalysis) comes from isotope effect studies that demonstrate that this enzyme gives V/K kinetic isotope effects that are intrinsic (Parkin & Schramm, 1984) and from pH studies.

The pH profiles of the K_m for AMP and K_{is} for formycin 5'-phosphate (a competitive inhibitor) are shown in Figure 6 [replotted from DeWolf et al. (1979)]. The profile for binding of formycin 5'-phosphate corresponds to that for the K_m for AMP, while the pH profile for V_{max} differs from the substrate and inhibitor binding profiles. This pattern can occur only if (a) the pK values for the dissociable groups that influence binding are the same on the substrate and competitive inhibitor, (b) substrate and inhibitor interact with groups with identical pK values on the enzyme, and (c) the rate constant for conversion of the enzyme–AMP complex to the reactive

complex(es) is insignificant relative to the dissociation of AMP from the enzyme. The pattern for the K_m of AMP and K_{is} for formycin 5′-phosphate corresponds until pH values above 9, where the pyrazolidine ring of formycin 5′-phosphate begins to ionize. These results with AMP nucleosidase provide further evidence that AMP is in equilibrium with the enzyme; thus, the commitment to catalysis is low for the enzyme–AMP complex.

The only kinetic data inconsistent with a simple rapid-equilibrium random mechanism are the small intercept effect of adenine (K_{ii} for adenine of 6.7 mM) in the product inhibition pattern. This pattern is consistent with adenine binding as a dead-end inhibitor with the enzyme–AMP complex. Since adenine is a planar, hydrophobic molecule, it may inhibit by insertion into a hydrophobic area of the protein that is accessible only when AMP is bound. The rapid-equilibrium mechanism is also consistent with previous studies with substrate analogues and direct binding studies (DeWolf et al., 1979; Schramm, 1976).

The dead-end enzyme–AMP–adenine complex is included in the proposed kinetic mechanism for AMP nucleosidase and is given in Scheme I together with the dissociation constants for each step in the reaction. The substrate saturation kinetics of the reverse reaction of AMP nucleosidase gave a sequential initial rate pattern and a kinetic dissociation constant for adenine that decreased from 0.35 to 0.1 mM as ribose 5-phosphate approached saturation. Likewise, the kinetic dissociation constant for ribose 5-phosphate decreased from 120 to 52 mM when the concentration of adenine was extrapolated to saturation. The kinetic patterns indicate synergism of substrate binding and give apparent dissociation constants that agree with those obtained from product inhibition studies. For example, the K_{ia} for adenine of 0.35 mM and K_{ib} for ribose 5-phosphate of 140 mM from the reverse reaction agree well with the K_{is} for adenine of 0.58 mM and K_{is} for ribose 5-phosphate of 110 mM from product inhibition studies. The good agreement of these constants indicates that the affinity of the enzyme for substrates is not strongly affected by the presence of 0.2 M potassium ion, which was necessary in measuring the reverse reaction. The maximum rates in the forward and reverse reactions are also obtained from the kinetic analysis and are indicated in Scheme I. Turnover numbers are expressed per enzyme dimer of approximately M_r 110 000, since the functional unit of the AMP nucleosidase hexamer is the dimer (Schramm & Fullin, 1978; Schramm, 1976; Schramm & Reed, 1980). The enzyme is written as E in Scheme I and represents the enzyme saturated with MgATP, the allosteric activator, since all kinetic studies used near-saturating concentrations of the activator. The upper pathway of product release is preferred to the lower pathway by the ratio of dissociation constants for adenine and ribose 5-phosphate release from the ternary complex. This calculation indicates a 520-fold preference for ribose 5-phosphate being released first. Thus, while the mechanism is formally random, only 1 of 520 events occurs with adenine release preceding ribose 5-phosphate release.

Additional evidence for the conversion of central complexes as the rate-limiting step comes from the lack of exchange of labeled adenine or ribose 5-phosphate into AMP during the steady-state period of AMP hydrolysis. Rate-limiting release of adenine or ribose 5-phosphate or a rate-limiting hydrolysis of a covalent E–ribose 5-phosphate complex would be expected to allow significant rates of reverse exchange into AMP. For example, NAD glycohydrolase catalyzes the exchange of nicotinamide into the substrate NAD at 0.3 of the initial reaction rate during initial rate kinetic (Schuber et al., 1976). Experiments that would have detected exchange of adenine or ribose 5-phosphate into AMP at 10^{-3}–10^{-4} of the initial rates indicated that such exchange does not occur with AMP nucleosidase. Although negative results cannot be used as proof for a mechanism, these results add further support for the mechanism proposed above.

Equilibrium Constant for AMP N-Glycoside Hydrolysis. Direct measurement of the equilibrium position gave a K_{eq} of approximately 280 M at concentrations of ribose 5-phosphate near 2 M and of approximately 170 M at concentrations of ribose 5-phosphate below 1.4 M. The lower value is considered to be more accurate since it is not possible to exceed the AMP concentration that defines the equilibrium constant when the equilibrium is approached with adenine and ribose 5-phosphate as substrates. At 2 M ribose 5-phosphate, the potassium counterion is present at 4 M, which may cause time-dependent inactivation of the enzyme, giving the false appearance of having reached equilibrium.

The K_{eq} can also be estimated from the Haldane (1930) relationships when the appropriate kinetic constants are known. For the mechanism in Scheme I, the Haldanes are

$$K_{eq} = V_1 K_{adenine} K_{i,ribose\text{-}5\text{-}P} / (V_2 K_{AMP})$$

and

$$K_{eq} = V_1 K_{i,adenine} K_{ribose\text{-}5\text{-}P} / (V_2 K_{AMP})$$

Substitution of average values from Table I gives Haldanes of 36 and 73 M, respectively, in reasonable agreement with the directly determined K_{eq} of 170 M. The necessity of using near-saturating concentrations of adenine and ribose 5-phosphate concentrations in excess of 1 M to generate measurable concentrations of AMP may lead to a slight overestimation of the K_{eq} when determined directly (i.e., failure to reach complete equilibrium). With K_{eq} values in the range of 36–170 M, the corresponding Gibbs free energies for N-glycosidic bond hydrolysis of AMP are −0.9 to −1.3 kcal/mol with H_2O assumed to be at standard conditions (1 M). From these results, the best estimate of a $\Delta G°$ for AMP hydrolysis is −1.1 ± 0.2 kcal/mol.

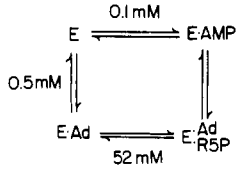
An equilibrium constant for the C–N glycosidic bond hydrolysis of AMP or other nucleotides has not been reported. However, the K_{eq} for this reaction may be estimated from the known free energies of the following chemical reactions:

AMP + PP_i → Ad + PRPP	$\Delta G°$ = 6.8 ± 0.5 kcal/mol	(Kornberg et al., 1955)
AMP + PRPP → ATP + R5P	$\Delta G°$ = 2.0 ± 0.5 kcal/mol	(Switzer, 1969)
ATP + H_2O → AMP + PP_i	$\Delta G°$ = −9.0 ± 0.5 kcal/mol	(Alberty, 1968)
AMP + H_2O → Ad + R5P	$\Delta G°$ = −0.2 ± 1.5 kcal/mol	

Although the relative error is high in this estimation, the $\Delta G°$ = of −0.2 kcal/mol corresponds to a K_{eq} = [adenine][ribose 5-phosphate]/[AMP] of 77 M, (with a range from 6 to 939 M) in good agreement with the experimental value of 170 M and the values of 36 and 73 M from the Haldane relationships.

Evidence for Enzyme-Directed H_2O. The chemistry of C–N bond hydrolysis involves attack of H_2O on C-1 of ribose 5-phosphate. From steady-state kinetic studies it is not possible to distinguish whether a covalent intermediate such as E–ribose 5-phosphate is formed, which is then attacked by H_2O, or whether a concerted reaction occurs in which a stabilized carboxonium intermediate is formed, which is then attacked by solvent or enzyme-directed H_2O. However, it is possible to distinguish between attack by a bulk solvent nucleophile

Scheme II: Thermodynamic Box for Major Reaction Pathway of AMP Nucleosidase[a]



[a] The constants are dissociation constants for enzyme–substrate and enzyme–product complexes.

as opposed to an enzyme-bound H_2O molecule. If the enzyme intermediate is attacked by solvent nucleophile, methanol should react more favorably than H_2O, since in many reactions methanol is the better nucleophile (Jencks 1969). A study of this type has been done with the calf spleen NAD glycohydrolase (Pascal & Schuber, 1976) from which it was demonstrated that methanol reacts 100 times better than H_2O and resulted in retention of configuration. This experiment established the covalent nature of the intermediate for NAD glycohydrolase and demonstrated that the final nucleophilic displacement occurs with solvent molecules.

Similar experiments with AMP nucleosidase gave no evidence for incorporation of labeled methanol into products and indicate that the site of attack by H_2O is not freely accessible to methanol. Since methanol would not be expected to interact with a specific binding site for H_2O, these results indicate that hydrolysis of AMP by AMP nucleosidase involves a specific binding site for H_2O.

It has been proposed that the enzymatic and nonenzymatic hydrolysis of some glycosides (Capon, 1969; Imoto et al., 1972), nucleosides (Jordon & Niv, 1977), and nucleotides (Bull et al., 1978) proceeds via a stabilized carboxonium ion intermediate. The results reported here do not rule out this possibility. However, a species so formed would not be free to interact with bulk solvent and would remain enzyme-bound until quenched by the attack of the enzyme-bound water molecule.

Energetics of Reaction Catalyzed by AMP Nucleosidase. The Gibb's free energy equation

$$\Delta G^\circ = -2.3RT \log K_{eq}$$

and the van't Hoff equation

$$\log \frac{K_{eq}^2}{K_{eq}^1} = \frac{\Delta H^\circ}{2.3\ R} \frac{T_2 - T_1}{T_2 T_1}$$

can be used to determine the thermodynamic functions for any reactions that can be described by equilibrium constants. Once the free energy and enthalpy terms have been evaluated, the contribution of entropy may be determined from the expression:

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$$

During catalysis, an equilibrium is established between the Michaelis complex and the transition-state complex. The enthalpy for this transition ($\Delta H^\ddagger$) can be obtained from the plot of log (V_{max}/T) as a function of $1/T$. The entropic value associated with transition-state formation can be calculated as described by Low et al. (1973), and the activation energy (E_a) can be calculated from the expression:

$$E_a = \Delta H^\circ + RT$$

The mechanism for AMP hydrolysis by AMP nucleosidase can also be written as a thermodynamic box as shown in Scheme II, where the constants represent dissociation constants for each of the steps that involve substrate or product addition or release. The equilibrium for the conversion of E·AMP to E–adenine–ribose 5-phosphate (hydrolysis) can be estimated from the relationship

$$\frac{[\text{E–adenine–ribose-5-P}]}{[\text{E·AMP}]} = \frac{K_{AMP}K_{eq}}{K_{i,adenine}K_{ribose\text{-}5\text{-}P}} = \frac{V_1}{V_2}$$

which is readily obtained from the equilibrium constants of Schemes I and II and the Haldane relationship. The ratio of E–adenine–ribose-5-P/E–AMP is calculated to be 650 from K_{eq} and the substrate and product dissociation constants when H_2O is taken as unity for calculation of K_{eq}. From the V_1/V_2 relationship, the ratio is 310, in good agreement with the ratio calculated from the kinetic constants. The average value of 480 is a reasonable estimate of the equilibrium of the enzyme-bound reactants. The ΔG° for hydrolysis of enzyme-bound AMP is thus −3.7 kcal/mol. This ratio is unlike the equilibrium of substrate–product pairs on transferases, which often exhibit ratios near 1 [e.g., Cohn & Reed (1982)], but is similar to the allosteric methylenetetrohydrofolate reductase, which also has an internal equilibrium far from unity (Vanoni & Matthews, 1984).

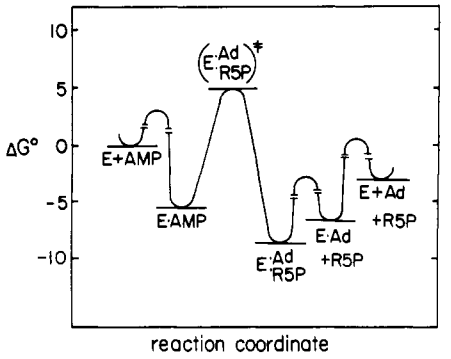


FIGURE 7: Energy diagram for the reaction catalyzed by AMP nucleosidase. The diagram is based on the equilibrium free energy values listed in Table III and the energies of activation derived from Figure 5. The energy level of the enzyme and substrate (E + AMP) has been arbitrarily assigned to be zero, at 30 °C, pH 8.0, and other energy levels are measured in reference to E + AMP, at a 1 M standard state for each reactant including H_2O. The horizontal lines represent energy changes that have been experimentally established. The broken lines indicate that the energy of activation for the formation of the complex is not established. AMP nucleosidase saturated with MgATP is written as E. Ribose 5-phosphate and adenine are written as R5P and Ad, respectively. Values of ΔG° on the ordinate are kcal/mol.

The free energy, enthalpy, and entropy values for the reaction catalyzed by AMP nucleosidase are given in Table III, and the reaction coordinate diagram for the enzyme is given in Figure 7. Analysis of the reaction catalyzed by AMP nucleosidase by these methods assumes that the reaction is rapid-equilibrium as indicated in Scheme I. The order of product release is ribose 5-phosphate followed by adenine for this analysis, since that pathway predominates. Estimates for the energy levels of the intermediates of the reaction catalyzed by AMP nucleosidase were derived from the kinetic and equilibrium data and are presented in Figure 5. The temperature dependence of V_{max} permits estimation of the energy of activation for the transition-state complex. The energy of activation values for formation of the other intermediates such as E·AMP, E·Ad, and free E cannot be established without measurement of individual rate constants for formation or dissociation of the complexes. It is possible to place limits on some of these constants on the basis of the kinetic isotope effects with AMP nucleosidase, which demonstrate that V/K intrinsic kinetic isotope effects are observed with AMP as substrate (Parkin & Schramm, 1984). The upper limit for individual rate constants is therefore governed by the diffusion

constant while the lower limit is regulated by the magnitude of the rate constant, which would obscure the kinetic isotope effect. If the reaction is written

$$E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}AMP \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{:}{}^{Ad}_{R5P} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}Ad \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E$$

during initial rate studies k_6 and k_8 will be zero, k_3 is 62 s^{-1}, and k_4 is 0.2 s^{-1}. For the rapid-equilibrium reaction of Scheme I, release of ribose 5-phosphate (k_5) is the first irreversible step for V/K considerations. Values for k_2 and k_5 that increase the energy of activation required for substrate addition or product release to absolute values larger than that required for conversion of E·AMP to $(E{\cdot}Ad{\cdot}R5P)^{\ddagger}$ can be eliminated since these would obscure the kinetic isotope effects (Northrop, 1981). A large energy barrier between E + Ad and E·Ad can also be eliminated, since this would permit exchange of ribose 5-phosphate into AMP during initial rate measurements. Thus, the reaction-coordinate diagram in Figure 7 is a reasonable estimate of the reaction profile.

The large negative entropy of AMP binding at temperatures above 25 °C indicates that substrate binding causes substantial increase in the order of the complex. Entropic changes of this magnitude upon substrate binding are normally associated with a more closed structure, for example, as a result of increased hydrogen bonding in protein–protein and substrate–protein interactions (Laidler & Peterman, 1979). Addition of AMP to AMP nucleosidase is known to prevent dissociation of the enzyme by phosphate (an allosteric inhibitor) and by decreased ionic strength (Schramm & Fullin, 1978), consistent with the calculated entropy of AMP interaction (Table III). Substrate specificity studies with AMP nucleosidase (DeWolf et al., 1979) also suggest that multiple enzyme–substrate contacts are required for productive binding to the enzyme. The AMP binding stoichiometry of AMP nucleosidase requires two identical polypeptide chains per binding site. If substrate binding involves contact with two peptide chains, the subunit structure will be stabilized, bringing a large entropic change. Cross-linking or X-ray diffraction studies will be needed to test this hypothesis.

Below 20 °C, the enthalpy of activation for catalysis and substrate binding change considerably, indicating that a temperature-dependent change in protein conformation occurs between 20 and 25 °C. The energy of activation for formation of the transition state is 11.4 kcal/mol at 30 °C and increases to 19.1 kcal/mol at 10 °C. At lower temperature, the enzyme has an additional energy barrier to formation of the transition state. In contrast, the enthalpy of substrate binding decreases at the enzyme conformation below 20 °C. The $\Delta G°$ for AMP binding changes from −5.5 kcal/mol at 30 °C to −5.7 kcal/mol at 10 °C, while the enthalpic component decreases from 12.7 to 6.4 kcal/mol and the entropic component changes from −23.8 kcal mol^{-1} deg^{-1} at 30 °C to −2.5 kcal mol^{-1} deg^{-1} at 10 °C. The loss of the entropic component is consistent with the cold enzyme existing in a closed conformation in which the substrate site conforms more closely to the solution structure of the substrate. This causes the substrate to bind better (K_m of 37 μM) but does not strain the substrate as much as does binding at 30 °C. Thus, the enthalpy of activation increases to 19.1 kcal/mol, since there is a decreased probability of reaching the transition state from this conformation.

Conclusions. The kinetic mechanism of AMP nucleosidase from *Azotobacter vinelandii* is rapid-equilibrium with random product release but with a strong preference for release of ribose 5-phosphate before adenine. No evidence exists for the formation of a covalent intermediate with ribose 5-phosphate. The water molecule that reacts with C-1′ of AMP is likely to be enzyme-bound. The reaction is reversible, and the K_{eq} for AMP hydrolysis is 170 M. The equilibrium position for enzyme-bound substrates and products favors hydrolysis. The enzyme exists in two different configurations at lower and higher temperatures. Substrate binding at higher temperatures includes a strong entropic component, which is lost at lower temperatures.

Registry No. AMP, 61-19-8; R5P, 4300-28-1; Ad, 73-24-5; AMP nucleosidase, 9025-45-0; formycin 5′-phosphate, 13270-66-1.

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