Effects of Allosteric Activation on the Primary and Secondary Kinetic Isotope Effects for Three AMP Nucleosidases*

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Kinetic isotope effects (V/K) were measured with AMP nucleosidases isolated from Azotobacter vinelandii, from a V_{max} mutant enzyme of A. vinelandii and from Escherichia coli. Specifically labeled AMP substrates were used to measure 3H secondary and 14C primary kinetic isotope effects on the N-glycosidic bond hydrolysis of AMP in the presence and absence of the allosteric activator, MgATP. Use of the three enzymes, variable MgATP concentration, a poor substrate (dAMP), and variable pH has allowed determination of the isotope effects over a 5000-fold range in the catalytic turnover number. The primary kinetic isotope effects were 1.025 ± 0.004 and 1.041 ± 0.006 for the native A. vinelandii enzyme and mutant enzyme, respectively, and were independent of MgATP concentration. The E. coli AMP nucleosidase had a primary isotope effect of 1.019 ± 0.003 which was also independent of MgATP concentration. The secondary kinetic isotope effect decreased from 1.068 ± 0.003 to 1.045 ± 0.002 for the native enzyme from A. vinelandii as the concentration of MgATP increased from 0 to 500 μM. The secondary isotope effect of the mutant enzyme remained constant at 1.088 ± 0.005 as the MgATP concentration increased from 0 to 500 μM. The secondary isotope effect of the E. coli enzyme showed a similar pattern to that of the native enzyme, decreasing from 1.087 ± 0.003 to 1.050 ± 0.003 as the enzyme was saturated with MgATP at a constant concentration of 100 μM. Saturation with AMP in the absence of MgATP gave similar results and suggested that AMP can cause the allosteric transition. Both the primary and secondary isotope effects for the native enzyme from A. vinelandii remained constant as the pH was varied in the absence of MgATP. Secondary isotope effects with a poor substrate, dAMP, were 1.08 for both the mutant and wild type enzymes from A. vinelandii in the presence of allosteric activator. In the native enzyme, this isotope effect was independent of MgATP concentration.

The relative insensitivity in the magnitude of observed isotope effects to pH, allosteric activator, the mutant enzyme, and a poor substrate (dAMP) indicate that intrinsic isotope effects are being expressed. The data are interpreted in terms of a single rate-limiting transition state for hydrolysis of the N-glycosidic bond, although other mechanisms cannot be eliminated. Using this model, the transition states of the native A. vinelandii and E. coli enzymes exhibit properties of both dissociative and associative mechanisms but become more associative as the allosteric activator becomes saturating. The transition state of the mutant enzyme is more dissociative and is not altered by increasing concentrations of MgATP. Likewise, the transition state for dAMP hydrolysis does not change as a function of allosteric activation. A mechanism is proposed in which the allosteric activator causes the incipient nucleophile to be moved closer to the C-1' of AMP and induces additional strain on the N-glycosidic bond.

AMP nucleosidase catalyzes the hydrolysis of the N-glycosidic bond of AMP to yield adenine and ribose 5-PO4. MgATP is an allosteric activator of AMP nucleosidase and increases the V_{max} for AMP hydrolysis by approximately 300-fold for the enzyme from Azotobacter vinelandii (1). The purpose of this work was to use heavy atom isotope effects to study the transition state complexes of several AMP nucleosidases and to determine whether the allosteric activator exerts its influence on the nature of the transition state. AMP nucleosidases from A. vinelandii provide a convenient way of altering turnover number since MgATP is a V_{max} activator (1) and a mutant enzyme has been characterized with a V_{max} of only 1% of the native enzyme (2). The AMP nucleosidase from Escherichia coli is also activated by MgATP, but for this enzyme the effector lowers the K_{m} value for AMP (3). Together, these enzymes provide an opportunity to examine the effects of allosteric transitions in reactions which can be examined by both primary and secondary kinetic isotope effects.

Model studies of N-glycosidic bond hydrolysis are provided by earlier literature reports of secondary isotope effects on the acid-catalyzed solvolysis of nucleosides and by model studies of primary isotope effects in N-glycoside bond cleavage and are discussed in the accompanying report (4). The acid-catalyzed hydrolysis of purine and pyrimidine nucleosides is believed to proceed via an SN1 mechanism with considerable oxycarbonium ion characteristic in the transition state (4-9), giving rise to a secondary 1H/1H kinetic isotope effect of 1.24, and a primary 13C/13C isotope effect of 1.04 (4). Alkaline hydrolysis of adenine nucleosides is thought to proceed via a nucleophile attack on C-1 by the hydroxide ion (10) but is complicated by purine ring opening as a competing process. Thus, chemical precedence exists for both dissociative and associative hydrolysis of the N-glycosidic bond of purine nucleosides.

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Several enzymes involved in the cleavage of N- and O-glycosidic bonds have been shown to act via transition states which have considerable dissociative (Sn1) characteristic. These enzymes include lysozyme (11, 12), several phosphoribosyltransferases (13), glycohydrolases (14), and purine nucleoside phosphorylase (15). These enzymes show relatively large secondary kinetic isotope effects. In contrast, columnic enzymes which catalyze similar reactions have small or insignificant deuterium secondary isotope effects when involved in the hydrolysis of N- or O-glycosidic bonds. Included are β-glucosidase (12), E. coli nitrocanthid monooctonucleotide glycohydrolase and pig brain and Neurospora crassa NAD glycohydrolases (14). Proposed explanations for low secondary isotope effects are: (i) substrates are bound irreversibly to the enzyme (large commitment to catalysis), (ii) the C-N or C-O bond is cleaved prior to the rate-limiting step, and (iii) the reaction proceeds via an associative mechanism. The proposed explanation for the small isotope effect for both the NAD and NAD glycohydrolases is that C-N bond cleavage is not involved in the rate-limiting step (14). For the hydrolysis of phenyl β-D-glucopyranoside by β-glucosidase, the secondary isotope effect is attributed to an associative mechanism (12).

These considerations have led to the proposal that kinetic isotope effects could provide a valuable technique to determine the effect of allosteric activation on the isotope-sensitive steps of enzymatic catalysis (16). Enzymes from different sources catalyzing the same reaction are expected to have similar transition states (17). In addition, isotope effects should prove useful to establish the nature of the catalytic defect in enzymes which have been rendered less active by structural mutations resulting from genetic manipulation. All of these applications of isotope effects have been exploited in the results reported here. The secondary H and primary 14C kinetic isotope effect of three AMP nucleosidases on the hydrolysis of AMP have provided evidence that the reaction has characteristics of both dissociative and associative mechanisms depending on the enzyme source and the degree of saturation with the allosteric activator (MgATP). The A. vinelandii and E. coli nucleosidases have significant dissociative characteristic at low concentration of MgATP but show increasing associative character as the allosteric transition is induced. The isotope effects with mutant AMP nucleosidase from A. vinelandii suggest a more dissociative mechanism. These results indicate that the increased rate of hydrolysis caused by the allosteric activator could be due in part to a change in mechanism in which the transition state becomes more associative.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adenosine 5'-monophosphate with H or 14C substituted at the C-1' or C-5' of ribose 5-PO4 was prepared as described in the preceding paper (4). Deoxyadenosine 5'-monophosphate was synthesized from labeled AMP using bacterial ribonucleotide reductase as described previously (4, 18). The native and mutant AMP nucleosidases from Azotobacter vinelandii were isolated as described previously (2, 19). The AMP nucleosidase from E. coli was isolated as described previously (3) except that a Sephadex G-200 column (3.5 × 65 cm) equilibrated with 0.1 M Tris-HCl, pH 8, 2 mM AMP, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM KCl and 3 μM phenylmethylsulfonyl fluoride was substituted for the hydroxylapatite column. The enzyme was eluted with the same buffer and activity was determined and enzyme was concentrated as described previously (2). The concentrated fractions were used as the source of E. coli AMP nucleosidase for kinetic isotope effect studies. All enzymes were purified to near homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The specific activities of homogeneous AMP nucleosidases from A. vinelandii, E. coli, and the mutant enzyme from A. vinelandii are 34, 24, and 0.3 μmol/min/mg of protein, respectively.

**Methods**—The kinetic isotope effect measurements were conducted as described in the previous paper (4) except for the following modifications. Labeled AMP substrates with 3H and 14C in the desired positions were combined with 0.1 M triethanolamine-HCl, pH 8, 1 mM MgCl2, variable ATP (0-0.5 mM), and variable AMP (0.2-50 mM). Reactions were initiated by the addition of enzyme and terminated by the addition of EDTA and formycin 5'-PO4 to give final concentrations of 1.5 and 0.15 mM, respectively. The isolation of ribose 5-PO4 and determination of the kinetic isotope effect was described in the preceding paper (4).

**RESULTS**

**Controls for Isotope Effect Measurements**—Control experiments described in the accompanying paper established the validity of using [1'-3H] and [1'-14C]AMP, adenosine, and inosine to determine the kinetic isotope effects on nonenzymatic cleavage of the N-glycosidic bond (4). To further validate these methods for the enzymatic hydrolysis of AMP, additional controls were established. The kinetic isotope effect was determined enzymatically using [5'-H]AMP and [5'-14C]AMP, where both labels are in isotopically nonsensitive positions relative to glycosidic bond cleavage. The results in Table I indicated [5'-14C]/[5'-H] isotope effects of 0.994 ± 0.002 and 0.993 ± 0.005 when both labels are in the 5' position of AMP, in the presence and absence of MgATP. An additional control is the comparison of the original ratio of [H]AMP/[14C]AMP in the substrate compared to the [H] ribose 5-PO4/[14C]ribose 5-PO4 following complete hydrolysis of the substrate. The ratios of 14C/H in AMP and ribose 5-PO4 were the same within experimental error. A final control to establish the validity of isotope effect measurements is to establish that the measured isotope effects decrease according to the fraction of the substrate converted to ribose 5-PO4. Both the primary and secondary isotope effects decrease to unity as the extent of substrate hydrolysis approaches 100% (Fig. 1). These controls demonstrate that the kinetic isotope effects follow the expected patterns and indicate that these techniques are valid for measuring the enzymatic isotope effects.

**Effect of pH on Primary and Secondary Isotope Effects**—The 14C primary and 3H secondary isotope effects were measured at pH 6, 7, and 8, a pH range which causes a 40-fold increase in the rate of AMP hydrolysis for the A. vinelandii enzyme. Under the conditions of the experiment (Fig. 2), the primary 12C/14C and secondary 1H/3H isotope effect was 1.025 ± 0.003 in the absence of MgATP. These values are not significantly different, and the average of 12C/14C isotope effect measurements was 1.025 ± 0.002.
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TABLE I

Control experiments for determination of kinetic isotope effects

In experiments I-III the reaction mixture contained 0.1 M triethanolamine-HCl, pH 8.0, 1 mM MgCl₂, 1.2 mM AMP, native AMP nucleosidase from A. vinelandii, and the indicated substrates at 30 °C. In experiments I and II, the reaction was stopped with EDTA and formycin 5'-P, and the isotope effect was determined by comparing the $^{14}$C/$^3$H ratio of ribose 5-P0₄ at 10–20% of AMP hydrolysis to that at 0 or 100% of AMP hydrolysis as described under "Experimental Procedures." In experiment III AMP hydrolysis was allowed to proceed until >99% of AMP was converted to ribose 5-P. The ratio of $^3$H/$^{14}$C is for ribose 5-P0₄ eluted from the charcoal column. Experiment IV is the $^{3}$H/$^{14}$C ratio of the labeled AMP substrates used for experiment III. The scintillation vials contained ~1 nmol of labeled AMP in addition to the same amounts of ribose 5-P0₄ and H₂O concentration used in other isotope effect measurements.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate(s)</th>
<th>AMP hydrolyzed</th>
<th>Ratio $^3$H/$^{14}$C</th>
<th>Observed KIE$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>[5'-$^3$H]AMP and [5'-$^{14}$C]AMP + 0 MgATP</td>
<td>%</td>
<td>15</td>
<td>0.992 ± 0.003 (3)</td>
</tr>
<tr>
<td>II</td>
<td>[5'-$^3$H]AMP and [5'-$^{14}$C]AMP + 500 μM MgATP</td>
<td>18</td>
<td></td>
<td>0.994 ± 0.002 (3)</td>
</tr>
<tr>
<td>III</td>
<td>[5'-$^{14}$C]AMP and [1'-$^3$H]AMP + 500 μM MgATP</td>
<td>99</td>
<td>0.832 ± 0.003 (3)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>[5'-$^{14}$C]AMP and [1'-$^3$H]AMP</td>
<td>0</td>
<td>0.829 ± 0.002 (3)</td>
<td></td>
</tr>
</tbody>
</table>

*KIE, kinetic isotope effects.

Fig. 1. Kinetic isotope effect as a function of extent of reaction. The kinetic isotope effect was determined for AMP nucleosidase from A. vinelandii at pH 8. The AMP concentration was 1 mM and the MgATP concentration was 0.5 mM for the measurement of both the primary and secondary kinetic isotope effects. The data points with standard error bars were determined from a set of three kinetic isotope effect determinations. All other points were obtained from single isotope effect determinations. The lines drawn were theoretical lines obtained using the equation $IE = \ln (1 - f \times I_{obs})/\ln (1 - f)$ where $IE$ is the actual isotope effect, $I_{obs}$ is the observed isotope effect, and $f$ is the fraction of labeled AMP converted to product. The curves are based on the data points with the standard error bars.

Fig. 2. Adjusted kinetic isotope effect as a function of pH. The kinetic isotope effect was determined using native A. vinelandii AMP nucleosidase. The buffer contained 20 mM of each of 2-(N-morpholino)ethanesulfonic acid, piperazine-N,N'-bis(2-ethanesulfonic acid), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Tris, and cyclohexylaminoethanesulfonic acid (Ches) adjusted to the desired pH with KOH, HCl, and KCl to give 100 mM K⁺. The reaction mixtures contained 1.0 mM AMP and 1 mM MgCl₂ in a total volume of 0.3 ml. No ATP was added. The isotope effects were determined as described under "Experimental Procedures" and adjusted to 0% substrate hydrolysis using the equation in the legend of Fig. 1. The standard deviations of the mean value were from three determinations at each pH and the standard error bars represent two standard deviations from the mean.

The mutant nucleosidase from A. vinelandii has only 1% of the $V_{max}$ of the native enzyme but is otherwise similar in terms of $K_m$, $S_0.5$, molecular weight, and subunit structure (2). The mutant enzyme shows no change in either the primary $^{14}$C/$^3$H or the secondary $^3$H/$^{14}$C isotope effect as a function of increased rate of hydrolysis due to saturating MgATP (Fig. 4). The primary and secondary kinetic isotope effects are 1.041 ± 0.006 and 1.088 ± 0.005, respectively, which are significantly greater than the primary and secondary isotope effects for the native enzyme (Fig. 3). The mutant enzyme demonstrates an increase in rate in response to...
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FIG. 3. Adjusted kinetic isotope effect as a function of MgATP for native AMP nucleosidase from A. vinelandii. The reaction mixtures contained 1.2 mM AMP, 1 mM MgCl₂, 100 mM triethanolamine-HCl, pH 8.0, in addition to the indicated concentrations of ATP. The reaction was stopped with EDTA and formycin 5'-P0₄ and the kinetic isotope effect was determined as described under "Experimental Procedures." The observed isotope effects were adjusted to 0% of substrate hydrolyzed using the equation in the legend to Fig. 1. The lines were drawn by eye to fit the experimental points. The initial rate curve is shown for comparison and was determined previously using the same AMP concentration. Fig. 3B gives the change in secondary kinetic isotope effect (open circles) and the log of the initial reaction rate (open diamonds) as a function of MgATP.

MgATP which is qualitatively similar to the native enzyme; however, the rate-limiting step (or steps) is decreased by a factor of 100.

The primary and secondary kinetic isotope effects for AMP nucleosidase from E. coli as a function of MgATP activation gave a pattern similar to those observed for the native A. vinelandii AMP nucleosidase (compare Figs. 5 and 3). The average primary ¹³C/¹⁴C kinetic isotope effect was slightly smaller at 1.017 ± 0.003 and was not significantly changed by increasing MgATP to 500 μM, approximately 10 times the concentration required for half-maximum activation (Fig. 5). The secondary isotope effect in the absence of MgATP was

Fig. 4. Adjusted kinetic isotope effect as a function of MgATP for mutant AMP nucleosidase from A. vinelandii. The reaction mixture was the same as described in the legend to Fig. 3. The kinetic isotope effect was determined as described under "Experimental Procedures" and corrected to 0% substrate hydrolysis. The standard error bars represent two standard deviations from the mean of three independent determinations. The initial reaction rate curve was determined under the same conditions used to estimate the isotope effects. The lines are drawn by eye to fit the experimental points.

Fig. 5. Adjusted kinetic isotope effect as a function of MgATP for AMP nucleosidase from E. coli. The reaction mixture consisted of 0.2 mM AMP, 1.0 mM MgCl₂, 100 mM triethanolamine-HCl, pH 8.0, in addition to varying ATP. The reaction was stopped and the kinetic isotope effect determined as described under "Experimental Procedures" and corrected to 0% substrate hydrolysis. The standard error bars represent two standard deviations from the mean of three independent determinations. The initial rate curve was determined previously under the same conditions used to establish the isotope effects. The lines are drawn by eye to the experimental points.
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TABLE II

Kinetic isotope effect for native and mutant A. vinelandii AMP nucleosidase with [5'-3H]dAMP and [1'-3H]dAMP

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MgATP</th>
<th>Adjusted KIE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0</td>
<td>1.084 ± 0.003</td>
</tr>
<tr>
<td>Native</td>
<td>0.5</td>
<td>1.075 ± 0.002</td>
</tr>
<tr>
<td>Mutant</td>
<td>0.5</td>
<td>1.075 ± 0.002</td>
</tr>
</tbody>
</table>

of 1.084 ± 0.003 with dAMP in the absence of MgATP and one of 1.075 ± 0.002 when the allosteric activator was present at the near saturating concentration of 0.5 mM (Table II). These results differed substantially from the secondary isotope effects with AMP (compare Fig. 3 and Table II), by demonstrating a larger effect in the absence of MgATP and a smaller decline in response to the activator. The mutant enzyme gave a 'H/3H secondary isotope effect of 1.078 ± 0.003 with dAMP in the presence of 0.5 mM MgATP (Table II).

Studies with dAMP as substrate for the mutant enzyme in the absence of MgATP were not feasible since the reaction rate was too slow to permit sufficient product formation and subsequent analysis. (The reaction rate under these conditions was estimated to be less than 5 × 10⁻⁴ that of the native enzyme under optimum assay conditions.) The secondary isotope effect of 1.076 for the mutant enzyme with dAMP was not significantly different from that for the native enzyme under the same conditions (Table II).

DISCUSSION

General Considerations—Heavy atom isotope effects have provided considerable mechanistic information on the nature of transition state intermediates for chemical reactions (22-26). Interpretation of isotope effects for enzyme-catalyzed reactions is complicated by steady state factors which can reduce or obscure the actual or "intrinsic" isotope effect which occurs as a result of the chemical transformation in the transition state. Northrop (20) has considered the difficulty of assigning kinetic isotope effects to a single step in enzyme catalyzed reactions. In spite of these difficulties, enzymes which demonstrate kinetic isotope effects express at least a portion of the transition state chemistry as the observed isotope effect. By altering conditions such as pH which can influence the relative rate of the bond-breaking step or by the use of poor substrates, some success has been obtained in the expression of kinetic isotope effects (eg. Refs. 15 and 27). The AMP nucleosidase from A. vinelandii gives a large change in \( V_{\text{max}} \) as a function of allosteric activator and thus provides an additional method of altering rates for the expression of the kinetic isotope effect. In addition, the enzyme is a single substrate system, the reaction is irreversible under all normal assay conditions for AMP hydrolysis, poor substrates have been described, the enzyme gives large changes in rate as a function of pH, and a mutant enzyme is available which has a 100-fold decrease in the turnover number as its only kinetically detectable catalytic defect (2). All of these factors contribute to the ability to express intrinsic isotope effects.

Intrinsic isotope effects can be used to interpret the nature of the transition state, and changes in the transition state will be reflected as changes in the observed isotope effects. Studies...
with chemical systems have demonstrated secondary tritium isotope effects up to 1.38 for the interconversion of a carbon sp² center to an sp³ carboxonium with most measurements for such reactions falling in the range 1.10 to 1.30 (23). S₂-like mechanisms exhibit secondary tritium effects of 1.00 to 1.09 (25). The primary kinetic isotope effects are expected to be in the range of 1.00 to 1.08 for an S₁-like reaction with a carboxonium intermediate and from 1.09 to 1.15 for an S₂-like intermediate with a symmetric transition state (13). A significant primary isotope effect in a reaction with a low secondary deuteron isotope effect would unambiguously indicate an S₂-like mechanism since, as discussed below, steady state effects should equally obscure both secondary and primary effects. The model studies with acid-catalyzed hydrolysis of AMP in the accompanying paper give an example of a dissociative mechanism and its associated kinetic isotope effects.

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Isotope Effects with Native AMP Nucleosidase from A. vinelandii—Conditions which allow expression of near maximum catalytic activity of AMP nucleosidase from A. vinelandii result in significant primary and secondary kinetic isotope effects indicating that bond breaking is partially or wholly rate-limiting under these conditions. These results are in agreement with kinetic and thermodynamic studies which indicated that kinetically determined $K_a$ values are not significantly different from dissociation constants (21, 28). This result suggests a small commitment to catalysis following AMP binding and implicates catalysis or product release as rate-limiting steps. Kinetic studies indicate that both adenine and ribose 5-P-O$_4$ are poor inhibitors and suggest a random release of products. The release of ribose 5-P-O$_4$ is not likely to be rate-limiting based on the following criteria: labeled ribose 5-P-O$_4$ (or labeled adenine) does not exchange into AMP during the initial rate period of AMP hydrolysis even when both products are present; product inhibition by ribose 5-P-O$_4$ is weak, with apparent inhibition constants ($K_i$ slope and $K_i$ intercept) of greater than 0.1 mM; the $K_i$ for ribose 5-P-O$_4$ in the back reaction is not significantly different from the inhibition constants obtained for ribose 5-P in product inhibition studies. These results are consistent with the proposal that the rate-limiting step is glycosidic bond hydrolysis; however, the data do not eliminate the possibility that several kinetically significant complexes are involved in the hydrolytic step. The magnitude of the isotope effects are 1.047 for the secondary effect and 1.024 for the primary effect under experimental conditions which give rates of substrate hydrolysis near $V_{max}$. When MgATP is omitted from the reaction, the rate slows by approximately 300-fold and the secondary isotope effect increases to 1.068 with no significant change in the primary isotope effect. If the change in secondary isotope effect reflected a change in a step which obscures the chemical events of catalysis, the change should affect both primary and secondary effects. Although the expected change in the primary isotope effect would be small, decreasing from 1.029 to 1.028, it is possible that a change of this magnitude might be detected within the limits of error shown in Fig. 3. The change in isotope effect induced by the allosteric activator might also be attributed to two (or more) distinct chemical steps in catalysis. For example, the ¹⁴C-sensitive step could be fully expressed in the presence or absence of MgATP while the ¹³H isotope effect could be partially obscured by increasing the reaction rate with MgATP. The response of the secondary isotope effect to varying MgATP concentration argues against this possibility.

If MgATP is obscuring the step which expresses the secondary isotope effect, the decrease in the observed isotope effect should continue as the rate of catalysis increases. However in Fig. 3, the rate of hydrolysis doubles between 50 and 500 μM MgATP while the change in the secondary isotope is complete at 50 μM. This result, together with the independence of both the primary and secondary isotope effects as a function of pH (Fig. 2) is consistent with the postulate that intrinsic isotope effects are being expressed. A summary of the kinetic isotope effects obtained with the AMP nucleosidase from A. vinelandii is given in Table III together with those for the mutant and E. coli enzymes.

Although the present results do not completely rule out the possibility of multiple intermediate steps which give rise to the observed isotope effects, the results will be interpreted in terms of a single transition state structure which expresses intrinsic isotope effects until further experiments provide evidence for a more complex reaction sequence. Experiments designed to identify covalent intermediates in AMP nucleosidase have thus far been unsuccessful, although enzymes which catalyze similar reactions such as the NAD-glycohydrolases from calf spleen and bull semen (29, 30) are known to form covalent intermediates. Experiments similar to those which identified the covalent intermediates with the NAD-glycohydrolases failed to identify covalent intermediates from the AMP nucleosidase from A. vinelandii. No exchange of [¹⁴C]adenine into AMP occurs during initial rate conditions in the presence of a large excess of [¹⁴C]adenine or [¹⁴C] adenine and ribose 5-P-O$_4$. This experiment also demonstrates the functional irreversibility of the reaction. Experiments using both ⁷H and ⁴H secondary isotope effects and substrate substitution with both ¹³C and ¹⁴C in the same molecule will be needed to completely resolve these questions.

If intrinsic isotope effects are being measured, the transition state has aspects of both associative and dissociative mechanisms. Thus, the secondary ¹³H/¹⁴H isotope effect of 1.06–1.07 is in the range expected for an associative mechanism and well below that expected for a dissociative mechanism such as observed in the acid-catalyzed hydrolysis of AMP which gives an effect of 1.23 (4). And yet the primary ¹³C/¹⁴C isotope effect of 1.03 is in the range expected for a dissociative mechanism and is near that of 1.05 observed for the acid-catalyzed hydrolysis of AMP (4). Secondary isotope effects are attributed primarily to the difference in the out-of-plane bending modes for reactant and transition state (31). Reactant conditions which inhibit ionization or stabilization of the carboxonium intermediate of an S₂ transition state, or interference by the adenine leaving group or the incoming H$_2$O with the out-of-plane bending motion of the ³H, could be responsible for the relatively small secondary ¹³H/¹⁴H isotope effect (22). Carbon kinetic isotope effects are expressed to the maximum in S₂-like reactions where reaction coordinate motion is dominated by carbon and the incoming H$_2$O or hydroxyl ion and the leaving adenine group are equally separated in terms of force constants (e.g. Ref. 32). The addition of MgATP alters the transition state to cause a significant decrease in the secondary isotope effect with no significant change in the bonding to carbon. A model which is consistent with these findings is given in Model A in the absence of an allosteric activator, the transition state is a hybrid of associative and dissociative effects, strain being induced by numerous interactions with the adenine and ribose 5-P-O$_4$ rings (21). If the N-9-C-1' bond is partially broken before the attack by H$_2$O (or OH⁻) occurs, the observed isotope effects could be obtained. If the allosteric transition induced by MgATP simply moved the incipient H$_2$O (or OH⁻) closer to C-1', the
The results with dAMP can be interpreted with the same model except that the enzyme loses the ability to interact with the hydroxyl at C-2' which causes a 40-fold decrease in $V_{\text{max}}/K_m$ for AMP from 15 mM to approximately 1 mM, assuming that substrate saturation approximates a normal binding isotherm. These results are consistent with a transition state with little reaction coordinate motion by carbon as indicated by the small $^{13}$C/$^{12}$C primary isotope effect. The transition state appears to become more $S_N2$-like as a result of allosteric activation, as indicated by the decline in the magnitude of the $^{13}$C/$^{12}$C secondary isotope effect with no change in the primary isotope effect. The apparent change in transition state for an enzyme which gives only a $K_m$ (or $S_{0.5}$) response to activator, suggests that changes in substrate binding interactions are also influential in formation of the transition state. These relationships should be expected in mechanisms where induced strain is important in catalysis.

The secondary isotope effect is also altered as a function of substrate concentration with E. coli AMP nucleosidase. Isotope effects measured by the competitive technique give $V_{\text{max}}/K_m$ effects. These would not be expected to change as a function of substrate concentration unless substrate is involved in more than simple saturation of the catalytic site. In the E. coli AMP nucleosidase, two possibilities are the substrate induces the allosteric transition by saturation at substrate sites or that AMP, at high concentrations, binds to MgATP activation sites to cause the allosteric transition. Given the similarity between AMP and dAMP, and the same $V_{\text{max}}$ value with saturating AMP in the presence or absence of MgATP, the second possibility seems likely. Direct
binding studies will be required to distinguish between these alternative mechanisms.

CONCLUSIONS

The results of isotope effect studies with AMP nucleosidases have established that the catalytic step is a rate-limiting step. Alteration of reaction rates by several factors suggests that isotope effects are intrinsic. The allosteric modifier acts to alter the transition states of the two native enzymes, but not the mutant enzyme. The results provide the first indication that allosteric effectors can alter the nature of the transition state. Evidence is also provided that changes in enzyme catalytic rate are not necessarily accompanied by modified transition state structure. The allosteric effect is composed of changes to the transition state structure as well as inductive changes in the catalytic site which increase catalytic rate without necessarily changing the bond order of the bonds undergoing catalysis.

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