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 Vmax 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 120 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 nuclease 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 Vmax 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 turn-over number since MgATP is a Vmax activator (1) and a mutant enzyme has been characterized with a Vmax 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 Km 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-glycosidic 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/14C isotope effect of 1.24, and a primary 13C/14C isotope effect of 1.04 (4). Alkaline hydrolysis of adenine nucleosides is thought to proceed via a nuclease attack on C-1 by the hydroxide ion (10) but is complicated by pyrimidine opening as a competing process. Thus, chemical precedence exists for both dissociative and associative hydrolysis of the N-glycosidic bond of purine nucleosides.
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 phosphorybosyltransferases (13), glycohydrolases (14), and purine nucleoside phosphorylase (15). These enzymes show relatively large secondary kinetic isotope effects. In contrast, column 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 citotrimidane mononucleotide glycohydrolase and pig brain and Neurospora crassa NAD glycohydrolyses (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 glycohydrolyses 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 3H 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 character 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 3H or 14C substituted at the C-1′ or C-5′ of ribose 5-P04 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 triethanolamine-HCl, pH 8, 2 mM AMP, 0.1 mM diithiothreitol, 0.1 mM EDTA, 0.2 M KCl and 5 μM phenylmethanesulfonyl 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 MgC12, 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′-P04 to give final concentrations of 1.5 and 0.15 mM, respectively. The isolation of ribose 5-P04 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′-3H]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′-3H] isotope effects of 0.994 ± 0.002 and 0.993 ± 0.001 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 [3H]AMP/[14C]AMP in the substrate compared to the [3H] ribose 5-P04/[14C]ribose 5-P04, following complete hydrolysis of the substrate. The ratios of 14C/3H in AMP and ribose 5-P04 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-P04. 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 change in the rate of AMP hydrolysis for the A. vinelandii enzyme. Under the conditions of the experiment (Fig. 2), the primary 14C/13C isotope effect was 1.020 ± 0.002 and the secondary 1H/3H isotope effect at these pH values averaged 1.066 ± 0.003. There was no significant change in the primary or secondary kinetic isotope effect as a function of pH even though the rate of hydrolysis varied 40-fold between pH 6 and 7. Independence of kinetic isotope effects as a function of pH is an indication that intrinsic isotope effects are expressed (15, 20).

**Comparative Isotope Effects for Three AMP Nucleosidases**—The primary 12C/14C and secondary 1H/3H isotope effects for three AMP nucleosidases as a function of increasing rates of hydrolysis are shown in Figs. 3–6. Rate changes in Figs. 3 and 4 are due to MgATP acting as a Vmax allosteric activator, and in Fig. 5, MgATP activates the E. coli enzyme by lowering the Km for a saturating concentration of AMP. The results of Fig. 6 demonstrate the change in secondary isotope effect induced by substrate concentration in the E. coli enzyme in the absence of MgATP.

The primary kinetic isotope effect was 1.029 ± 0.003 in the absence of ATP and 1.024 ± 0.002 when MgATP was present at 500 μM. These values are not significantly different, and the average of 14C/13C isotope effect measurements was 1.025.
Isotope Effects for AMP Nucleosidases

**TABLE I**

Control experiments for determination of kinetic isotope effects

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate(s)</th>
<th>AMP hydrolyzed</th>
<th>Ratio $^3$H/$^1$C</th>
<th>Observed KIE$^a$ [5'-$^3$C]AMP</th>
<th>[5'-$^2$H]AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>[5'-$^3$H]AMP and [5'-$^1$C]AMP + 0 MgATP</td>
<td>%</td>
<td>15</td>
<td>0.992 ± 0.003 (3)</td>
<td></td>
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<tr>
<td>II</td>
<td>[5'-$^3$H]AMP and [5'-$^1$C]AMP + 500 μM MgATP</td>
<td>18</td>
<td></td>
<td>0.994 ± 0.002 (3)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>[5'-$^1$C]AMP and [5'-$^3$H]AMP + 500 μM MgATP</td>
<td>99</td>
<td>0.832 ± 0.003 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>[5'-$^1$C]AMP and [5'-$^2$H]AMP + 500 μM MgATP</td>
<td>0</td>
<td>0.829 ± 0.002 (3)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*KIE, kinetic isotope effects.

$^a$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 three experiments. The lines drawn were theoretical lines obtained using the equation $IE = \ln (1 - f \times IE_{obs})/\ln (1 - f)$ where $IE$ is the actual isotope effect, $IE_{obs}$ 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.

$± 0.004$ for the native A. vinelandii AMP nucleosidase (Fig. 3). In contrast, the secondary tritium isotope effect at 0 μM ATP of 1.068 ± 0.003 decreased substantially to 1.047 ± 0.002 at 500 μM MgATP. The decrease in the secondary isotope effect was complete at 50 μM MgATP (Fig. 3), which is near the kinetically determined $K_{m}$ of 40 μM for MgATP (1). The change in the secondary isotope kinetic effect is accompanied by a >100-fold increase in $V_{max}$ over this range of MgATP activation, while the final 2-fold increase in $V_{max}$ occurs above 50 μM MgATP occurring with no significant change in the isotope effect. The relationship between the change in the catalytic rate and the secondary isotope effect is more clearly demonstrated in a plot of the log of the initial reaction rate and the secondary kinetic isotope effect as a function of MgATP concentration (Fig. 3B).

The mutant AMP nucleosidase from A. vinelandii has only 1% of the $V_{max}$ of the native enzyme but is otherwise similar in terms of $K_{m}$, $V_{max}$, molecular weight, and subunit structure (2). The mutant enzyme shows no change in either the primary $^3$C/$^1$C or the secondary $^1$H/$^2$H 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
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 standard error bars represent two standard deviations from the mean of three independent determinations. The initial reaction rate curve is shown for comparison and was determined previously using the same AMP concentration. The lines were drawn by eye to fit the experimental points.

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 $^{13}C/^{12}C$ 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 reaction 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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1.087 ± 0.003, significantly larger than that for the native A. vinelandii enzyme (compare Figs. 3 and 5). The secondary isotope effect decreases significantly as a function of increasing MgATP concentration. At a constant concentration of 0.2 mM MgATP, the $^{1}H/H$ secondary isotope effect decreased from 1.087 ± 0.003 to 1.050 ± 0.003 as MgATP increased from 0 to 500 μM. In the absence of ATP, the secondary $^{1}H/H$ isotope effect decreased from 1.087 ± 0.003 to 1.087 ± 0.002 as the AMP concentration was increased from 0.2 to 50 mM. The apparent $K_{m}$ for AMP has been reported to be ~15 mM in the absence of MgATP (3), and this value is in good agreement with the initial rate measurements under conditions used for the isotope effect determinations (Fig. 6). The observed decrease in the secondary isotope effect was complete at 10 mM AMP, near the $K_{m}$ for AMP (Fig. 6). Likewise, the change in the secondary isotope effect for the E. coli enzyme was complete at 50 μM MgATP, near the apparent kinetic constant of 60 μM for activation by MgATP (Fig. 5). The change in the $^{1}H/H$ secondary isotope effect for E. coli AMP nucleosidase parallels the major changes in the rate of AMP hydrolysis. Thus, plots of log initial rate and secondary kinetic isotope effect (not shown) are similar to that shown in Fig. 3B.

Secondary Isotope Effect with dAMP—Deoxy-AMP is a poor substrate for AMP nucleosidase, giving a $V/K$ value which is 0.025 that for AMP, with the major factor being a decrease in the $V_{max}$ (21). A combination of [5'-$^{14}C$]dAMP and [1'-$^{3}H$]dAMP was synthesized from the corresponding nucleotides using ribonucleotide reductase as described under "Experimental Procedures." The deoxynucleotide was used to establish the secondary $^{1}H/H$ kinetic isotope effects for the native and mutant AMP nucleosidases from A. vinelandii.

The native enzyme gave a $^{1}H/H$ secondary isotope effect 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 $^{1}H/H$ secondary isotope effect of 1.076 ± 0.000 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$^{-6}$ 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 effects of steady state factors on intrinsic kinetic isotope effects and has emphasized 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 as a function of 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 $V_{max}$ 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). S₁₂ mechanisms exhibit secondary tritium effects of 1.00 to 1.09 (25). ¹³C/¹⁴C primary kinetic isotope effects are expected to be in the range of 1.00 to 1.08 for an S₁₂-like reaction with a carboxonium intermediate and from 1.09 to 1.15 for an S₂₂-like intermediate with a symmetric transition state (13). A significant primary ¹³C isotope effect in a reaction with a low secondary deuterium isotope effect would unambiguously indicate an S₂₂-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.

**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 $K_m$ 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 the rate-limiting steps. Kinetic studies indicate that both adenine and ribose 5-PO₄ are poor inhibitors and suggest a random release of products. The release of ribose 5-PO₄, is not likely to be rate-limiting based on the following criteria: labeled ribose 5-PO₄ (or labeled adenine) does not exchange into AMP during the initial period of AMP hydrolysis even when both products are present; product inhibition by ribose 5-PO₄ is weak, with apparent inhibition constants ($K_i$) for these two factors of 0.1 M; the $K_m$ for ribose 5-PO₄ 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 kinematically significant complexes are involved in the hydrolytic step. The magnitude of the isotope effects are 1.047 for the secondary isotope effect increases to 1.024 for the secondary isotope 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 ¹³C-sensitive step could be fully expressed in the presence or absence of MgATP while the ³H 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 ³H 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 effect 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 [³¹C]adenine and ribose 5-PO₄. This experiment also demonstrates the functional irreversibility of the reaction. Experiments using both ³H and ³¹H secondary isotope effects and substrate substituted with both ³H and ³¹C 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 ³H/³¹H isotope effect of 1.05–1.07 is in the range expected for an associative mechanism and well below that expected for a dissociative mechanism since 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). Complete conditions which inhibit ionization or stabilization of the carboxonium intermediate of an $S_{1}+1$ transition state, or interference by the adenine leaving group or the incoming H₂O with the out-of-plane bending motion of the ³H, could be responsible for the relatively small secondary ³H/³¹H isotope effect (22). Carbon kinetic isotope effects are expressed to the maximum in S₁₂ reactions where reaction coordinate motion is dominated by carbon and the incoming H₂O 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 which in the absence of the 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-PO₄ rings (21). If the N-9-C-1' bond is partially broken before the attack by H₂O (or OH⁻) occurs, the observed isotope effects could be obtained. If the allosteric transition induced by MgATP simply moved the incipient H₂O or (OH⁻) closer to C-1', the
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TABLE III
Summary of the kinetic isotope effects with AMP nucleosidases as a function of MgATP activation

<table>
<thead>
<tr>
<th>Substrate and isotope effect</th>
<th>Native A. vinelandii enzyme</th>
<th>Mutant A. vinelandii enzyme</th>
<th>E. coli enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ATP</td>
<td>+MgATP</td>
<td>No ATP</td>
</tr>
<tr>
<td>AMP $^{13}$C/$^{14}$C</td>
<td>1.029 ± 0.003</td>
<td>1.024 ± 0.002</td>
<td>1.041 ± 0.006</td>
</tr>
<tr>
<td>AMP $^{2}$H/$^{3}$H</td>
<td>1.066 ± 0.003</td>
<td>1.047 ± 0.002</td>
<td>1.088 ± 0.005</td>
</tr>
<tr>
<td>dAMP $^{2}$H/$^{3}$H</td>
<td>1.084 ± 0.003</td>
<td>1.075 ± 0.002</td>
<td>1.078 ± 0.003</td>
</tr>
</tbody>
</table>

FIG. 7. Hypothetical transition states for AMP nucleosidase. The left drawing represents the Michaelis complex which freely dissociates to reactants. The N attached to adenine represents N-9 of the purine ring and the N- C bond represents the N-glycosidic bond from N-9 to C-1' of AMP. The remainder of the molecule is omitted for clarity. R represents an enzyme group which interacts with the C-2' hydroxyl of AMP, and the reactive H$_2$O molecule is shown. The middle structure represents a hypothetical transition state in the absence of MgATP. R is weakly bonded to the C-2' hydroxyl, and the intermediate has both oxycarbonium character and an activated H$_2$O molecule acting as the incipient nucleophile. The structure on the right represents a hypothetical transition state in the presence of MgATP. The allosteric transition has compressed the enzyme which gives only a $V_{max}$ at 1000 times the rate of AMP. Although this mutation is consistent with the results, the other factors described above which interfere with the H-1' out-of-plane bending could also be involved in the altered secondary isotope effect.

Isotope Effects with Mutant AMP Nucleosidase from A. vinelandii —The enzyme which is a $V_{max}$ mutant gives increased primary and secondary isotope effects with AMP as substrate, and these values are insensitive to the allosteric activator. A mutation affecting a group (R in Fig. 7) which is proposed to interact with the 2'-OH of ribose could account for these results with the mutant enzyme. The transition state would be similar to that for the native enzyme with dAMP as substrate. In this case, the bonding to C-1' must be altered in the transition state since a significant increase occurs in the primary $^{13}$C/$^{14}$C isotope effect (Table III). The rate of catalysis by the mutant enzyme is increased by MgATP but this change is not accompanied by a change in the transition state. An analogous situation exists in the acid-catalyzed solvolysis of AMP and dAMP (4). In this case, the transition states are the same as deduced from the secondary kinetic isotope effect; but the inductive effect of the 2'-OH causes dAMP to hydrolyze about a thousand times more rapidly than AMP. A similar situation is likely to occur with the mutant enzyme. This effect could be generated by realignment of catalytic groups to improve the electron withdrawing ability of the adenine ring or the nucleophilicity of the attacking H$_2$O while maintaining the same bonding geometry to C-1' and H-1' in the transition state.

Isotope Effects with the E. coli Enzyme —The pattern of isotope effects with the E. coli enzyme indicates that a change in the transition state is an early effect of the allosteric activator. The degree of MgATP saturation which induces the major change in the transition state causes a decrease in the $K_m$ 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/$^{14}$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 $^{1}$H/$^{2}$H secondary isotope effect with no change in the primary isotope effect. The apparent change in transition state for an enzyme which gives only a $V_{max}$ (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 $V_{max}$/$K_m$ 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 sites. In the E. coli AMP nucleosidase, two possibilities are that 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 MgATP, and the same $V_{max}$ value with saturating AMP in the presence or absence of MgATP, the second possibility seems likely.
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 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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