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Transition State Stabilization by Deaminases: Rates of Nonenzymatic Hydrolysis of Adenosine and Cytidine[1]

LLOYD FRICK, JOHN P. MAC NEELA, AND RICHARD WOLFENDEN

Department of Biochemistry, 231 H, University of North Carolina, Chapel Hill, North Carolina 27514

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Nonenzymatic rates of hydrolytic deamination of adenosine and cytidine by acids and bases analogous to side chains of naturally occurring amino acids are compared with the rates of uncatalyzed deamination in water and with the rates of the hydroxide- and hydrogen ion-catalyzed reactions. For adenosine, hydroxide ion is an effective catalyst, with a second-order rate constant of 7.5×10^{-6} M^{-1} s^{-1} at 85°C and an energy of activation of 19.9 kcal/mol. Acid-catalyzed deamination of adenine proceeds with a second-order rate constant of 1.5×10^{-6} M^{-1} s^{-1} at 85°C. At concentrations of 1 M and at pH values corresponding to their respective pK_a values, dimethylamine, acetate, selenide, imidazole, phosphate, and zinc(II) do not enhance the rate of deamination of adenosine beyond that observed in water, and 2-mercaptoethanol produces only a modest rate enhancement. The uncatalyzed rate of adenosine deamination in water is 8.6×10^{-9} s^{-1} at 85°C: extrapolation to 37°C and comparison with k_{cat} for rat hepatoma adenosine deaminase yield a rate enhancement by the enzyme of approximately 2×10^{12}-fold. 1,6-Dimethyladenosine, the conjugate acid of which has a pK_a value much higher than that of adenosine, is not readily deaminated, suggesting that the uncatalyzed deamination of adenosine does not proceed by hydroxide ion attack on the rare protonated form of adenosine, but rather by attack on the neutral species. Deamination of cytidine is catalyzed most effectively by hydroxide ion, with a second-order rate constant of 4.5×10^{-4} M^{-1} s^{-1} at 85°C and an energy of activation of 28.5 kcal/mol. The uncatalyzed rate of deamination of cytidine in water, which also exhibits an energy of activation of 28.5 kcal/mol, is 8.8×10^{-8} s^{-1} at 85°C. Comparison of the rate extrapolated to 25°C with k_{cat} for bacterial cytidine deaminase gives a rate enhancement for the enzyme of 4×10^{11}-fold. The C-5 proton of the pyrimidine ring of cytidine does not exchange with solvent during alkaline hydrolysis, suggesting that deamination under these conditions does not involve prior addition of water across the 5,6 double bond.

INTRODUCTION

A fundamental property of any enzyme is its ability to increase the rate of a reaction above the spontaneous rate at which the reaction occurs in the absence of catalysis. This rate enhancement is due entirely to the enzyme's ability to bind and stabilize the transition state of the catalyzed reaction; accordingly, compounds resembling the altered substrate in the transition state prove to be very strong inhibitors of a variety of enzymes. The increase in affinity for the enzyme

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that occurs as the substrate proceeds from the ground state to the transition state therefore equals the increase in rate brought about by the enzyme. It is thus possible to estimate the upper limit of the K_{TX}, the binding affinity of an enzyme for an ideal transition state analog, by dividing the rate enhancement (k_{cat}/k_{non}) by the K_m of the enzyme [for a discussion of the limiting conditions for this calculation and its application to enzymes acting on multiple substrates or employing covalently bound intermediates, see Ref. (1)]. Measurement of the nonenzymatic rate is often difficult, because of the slow progress of biochemical reactions in the absence of enzymes, and because of the presence of competing side reactions. Thus, it is hardly surprising that k_{non} has been determined for only a few enzymatic reactions (see Discussion).

The hydrolytic deamination of adenosine and cytidine is catalyzed by a group of enzymes that play important physiological roles and appear to be responsible for the rapid degradation of many chemotherapeutic agents. Powerful reversible inhibitors, which may resemble high-energy intermediates in the deamination of adenosine and cytidine, have been discovered in nature and synthesized in the laboratory (*2–7*). These inhibitors, of which 2′-deoxycoformycin is the most potent, appear to have captured a portion of the enzyme's affinity for the transition state, but it is not possible to guess what fraction without information concerning the nonenzymatic rates of these reactions. The nonenzymatic hydrolysis of adenylate and cytidylate residues in DNA is also of interest as a possible source of spontaneous mutations (*8, 9*).

For these reasons, it seemed desirable to determine the spontaneous rate of hydrolytic deamination of adenosine and cytidine in water, and to examine the effectiveness of a variety of biologically occurring nucleophiles as potential catalysts. Thiols appeared to be of special interest, in view of evidence suggesting the presence of an essential thiol group in deaminases acting on cytidine, adenosine, and AMP (*10–12*), and because 6-chloropurine ribonucleoside reacts rapidly with sulfur nucleophiles (*13*). Selenide ion had been reported to displace ammonia from the 6-position of adenosine in aqueous pyridine (*14*). Because selenium is present in certain proteins, it seemed appropriate to include it in the present study.

METHODS

1-Methyladenosine and 6-methyladenosine were prepared by the methods of Jones and Robbins (*15*), and 1,6-dimethyladenosine by the method of Broom *et al.* (*16*). ^{14}C-labeled adenine, adenosine, and cytidine were purchased from New England Nuclear Corporation and purified before use by HPLC to remove traces of hydrolytic products. Other nucleosides and bases were obtained from Sigma Chemical Company, and their purity was established using HPLC.

Hydrolyses were conducted in sealed borosilicate tubes, with ionic strength adjusted to 1.5 using KCl. Purine nucleosides were analyzed by HPLC on a Whatman Partisil C-18 column, using a gradient of methanol in potassium acetate buffer (0.01 M, pH 4.76). Adenine hydrolysates were analyzed on the same column, using isocratic elution with 8% methanol. Cytidine and uridine were

separated on a cation-exchange column (Alltech Partisil 10 SCX) using isocratic elution with potassium phosphate buffer (0.02 M, pH 3.38). Where possible, the identity of peaks was confirmed by cochromatography with the authentic compound. Reactants and products were quantitated by scintillation counting or UV absorbance. Because the occurrence of side reactions caused a relatively rapid depletion of nucleosides, it was necessary to use the initial rates of reaction to calculate a rate constant.

The separate peaks of reactant and product were collected and quantified, and the rate constant was calculated using the equation $-kT = \ln A/A_0$, where A is the amount of reactant at time t, and A_0 is the initial amount (calculated by summing the amounts of reactant and product). Less than 30% of the nucleoside was consumed, and rates were fairly linear with time within this range, especially at lower temperatures.

Proton magnetic resonance spectra were recorded on a Varian EM-390 spectrometer. Uridine and cytidine controls, uridine and cytidine in 0.6 M NaOD, and cytidine with 300 units of *Escherichia coli* cytidine deaminase in phosphate buffer (pH 7.5, 0.1 M, evaporated once from D_2O), were incubated at room temperature for 21 days. The ratio of the integrated intensities of signals corresponding to the C-6 proton at 7.9 and 7.8 ppm to those of the C-5 proton at 6.1 and 6.0 ppm was determined.

RESULTS

The mixture of products obtained by alkaline hydrolysis of adenosine is shown in Fig. 1. Triaminopyrimidine arises as a major product from attack at the C-8 position of the purine ring (*17, 18*), and adenine arises from glycoside cleavage. Several unknown products were also observed, but inosine was easily resolved from them. Chromatograms of cytidine hydrolysates were much simpler (not shown).

Rate constants observed for hydrolytic deamination in the presence of various nucleophiles are shown in Table 1. Little catalytic activity was observed except in the case of hydroxide ion. The rate of inosine formation was proportional to hydroxide ion concentration over the range 0.01 to 1 M. Only moderate catalysis was observed with 2-mercaptoethanol. In acid solution, adenosine underwent glycosidic cleavage too rapidly to permit determination of its rate of deamination. We therefore examined the acid hydrolysis of adenine, the rate of cleavage of which under neutral conditions is similar to that of adenosine. Some enhancement of the rate of deamination was observed in acid. Cytidine was more readily deaminated at high temperatures than adenosine. Although hydroxide ion was once again the most effective catalyst, phosphate dianion (pH 7.1) was about 3% as active.

Arrhenius plots of adenosine and cytidine deamination in the presence of hydroxide ion are shown in Fig. 2. Energies of activation, obtained from linear regression analysis, were estimated to be 19.9 kcal/mole for adenosine and 28.5 kcal/mol for cytidine. Due to the slow rate of deamination and because side

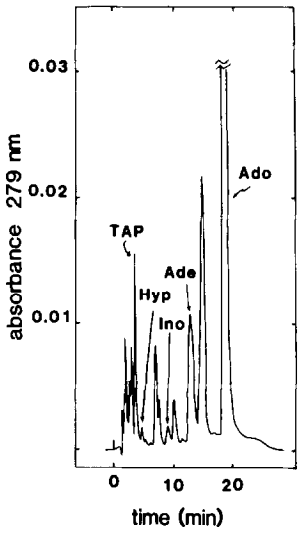

FIG. 1. HPLC of the products of adenosine hydrolysis. Adenosine (50 mM) was hydrolyzed in 0.1 M KOH at 85°C for 4 h and neutralized with an equal volume of 0.1 M HCl. Twenty microliters was loaded on a Whatman C-18 column (4.1 × 250 nm) and eluted at 2 ml/min with a 0–10% gradient of methanol over 10 min, beginning at 8 min. The aqueous phase was potassium acetate (10 mM), pH 4.8. TAP, triaminopyrimidine; Hyp, hypoxanthine; Ino, inosine; Ade, adenine; Ado, adenosine.

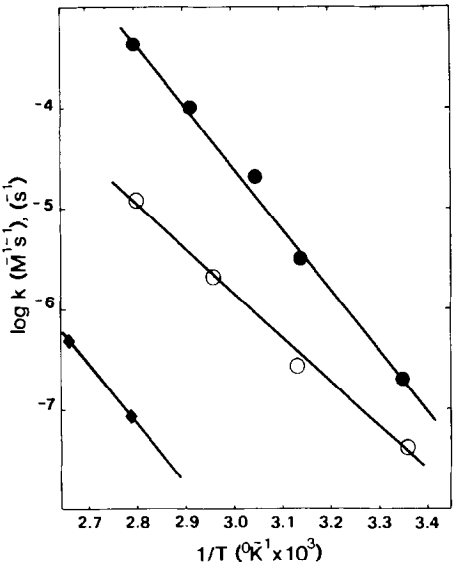

FIG. 2. Arrhenius plot for the deamination of adenosine and cytidine. Adenosine was incubated with 1 M KOH at 85, 65, 45, and 25°C, and inosine was isolated by HPLC. Cytidine was incubated with 1 M KOH at 85, 70, 55, 45, and 25°C, and uridine was isolated by HPLC. Uncatalyzed deaminations of cytidine were conducted at 100 and 85°C. Values for the second-order rate constant are given by ○ and ● for adenosine and cytidine, respectively; values for the first-order rate constant of the uncatalyzed deamination of cytidine are given by ◆.

TABLE 1

DEAMINATION RATE CONSTANTS OF NUCLEOSIDES AT 85°C

Substrate	Catalyst	Rate constant
Adenosine	KOH	7.5×10^{-6} M^{-1} s^{-1}
Adenine	HCl	1.5×10^{-6} M^{-1} s^{-1}
Adenosine	Mercaptoethanol	2.9×10^{-7} M^{-1} s^{-1}
Adenosine	Imidazole	$<10^{-7}$ M^{-1} s^{-1}
Adenosine	Dimethylamine	$<10^{-7}$ M^{-1} s^{-1}
Adenosine	Selenide ion[a]	$<10^{-7}$ M^{-1} s^{-1}
Adenosine	Acetate	$<10^{-7}$ M^{-1} s^{-1}
Adenosine	Phosphate dianion	$<10^{-7}$ M^{-1} s^{-1}
Cytidine	KOH	4.5×10^{-4} M^{-1} s^{-1}
Cytidine	Phosphate dianion	1.3×10^{-5} M^{-1} s^{-1}
Cytidine	Mercaptoethanol	2.0×10^{-7} M^{-1} s^{-1}
Cytidine	Dimethylamine	$<10^{-7}$ M^{-1} s^{-1}
Cytidine	Imidazole	$<10^{-7}$ M^{-1} s^{-1}
Adenosine	None	8.6×10^{-9} s^{-1}
Adenine	None	1.3×10^{-8} s^{-1}
Cytidine	None	8.8×10^{-8} s^{-1}

[a] Calf intestinal adenosine deaminase, when analyzed for selenium content, had less than 0.2 molar equivalent of this element.

reactions became severe at higher temperatures, it was not possible to estimate an energy of activation for the spontaneous deamination of adenosine. However, an Arrhenius plot of uncatalyzed cytidine deamination gave the same slope as the rates for the reactions catalyzed by hydroxide ion (Fig. 2). Rates of uncatalyzed deamination of both adenosine and cytidine, determined at neutral pH, were several orders of magnitude more rapid than would be the case if deamination involved attack on the low levels of hydroxide ion present, so that water was evidently the nucleophile under these conditions.

To determine whether the deamination of adenosine at neutral pH might proceed via rapid attack of hydroxide ion on protonated adenosine, the rate of alkaline hydrolysis of 1,6-dimethyladenosine was determined. This compound, by analogy to 1-methyladenosine (*19*), has a pK_a value several units higher than that of adenosine, so that the concentration of the protonated form would be expected to be correspondingly higher than that for adenosine. The dimethylated compound was chosen because of its freedom from ambiguities that would arise in 1-methyladenosine from the alkaline Dimroth rearrangement (*19*). Dimroth rearrangement of 1,6-dimethyladenosine leads to recovery of the starting material. In any event, no deamination of 1,6-dimethyladenosine was observed after 24 h in 1 M KOH at room temperature.

Under acidic conditions, deamination of cytidine has been shown to proceed via initial attack on the 5,6 double bond of the protonated nucleoside to yield a relatively unstable 5,6 saturated compound (*20*). To determine whether alkaline hydrolysis might occur by a similar mechanism, we looked for exchange of sol-

vent deuterium into the 5-position of uridine and cytidine in the presence of 0.6 M NaOD. NMR showed no detectable exchange after 3 weeks at room temperature, although a substantial amount of the cytidine had been converted to uridine. In D_2O buffered with phosphate, no detectable exchange was found to have occurred in the presence of a large excess of cytidine deaminase upon prolonged incubation.

DISCUSSION

Most previous studies of the decomposition of purines and purine derivatives in alkaline solution have focused on the more rapid ring opening and deglycosylation reactions (*17, 21, 22*). However, Karran and Lindahl (*8*) have reported an apparent rate constant of 4×10^{-8} s^{-1} for deamination of adenine residues in denaturated DNA at 110°C and pH 7.4, similar to the value that we observe for free adenosine. Jones *et al.* (*18*) reported rates of deamination of adenosine in KOH that can be used to calculate a second-order rate constant of approximately 1.1×10^{-4} M^{-1} s^{-1} at 100°C. The discrepancy between this value and the rate constant of 4.2×10^{-5} M^{-1} s^{-1}, which can be extrapolated from our data, can probably be ascribed to the inability of earlier TLC systems to distinguish inosine from the surprisingly complex mixture of products that result from adenosine hydrolysis.

The decomposition of cytidine has been examined previously in the presence of a variety of nucleophiles under mildly acidic conditions (*23–28*). Deamination of deoxycytidine by hydroxide ion, determined by following the change in UV absorbance of crude reaction products, gives a rate about one-third as rapid as our value for the deamination of cytidine by KOH (*29*). Sanchez and Orgel (*30*) provide data that can be used to calculate first-order rate constants for the disappearance of cytidine at neutral pH: 8×10^{-7} s^{-1} at 100°C and 1×10^{-8} s^{-1} at 60°C. These values are approximately double those reported here, although the energy of activation implied is similar. These differences in rate may also be due to the use of TLC to separate the products of the reaction. Lindahl and Nyberg (*9*) reported a rate constant of 2×10^{-7} s^{-1} for hydrolytic deamination of cytidylate in denaturated DNA at 95°C and an energy of activation of 29 kcal/mol, values similar to those we observe for the free nucleosides.

It is of interest that nucleophiles other than hydroxide ion were far less effective at catalyzing deamination than they were at catalyzing the hydrolysis of 6-chloropurine ribonucleoside (*13*). These results suggest that a double displacement mechanism for the enzyme reaction involving an active site amine, thiol, or selenium nucleophile may be unlikely on chemical grounds. Evidence that adenosine deaminase acts by direct water attack consists of its ability to catalyze the covalent hydration of pteridine (*31, 32*), and the structures of two very potent inhibitors of the enzyme, coformycin (*4, 33*) and 2′-deoxycoformycin (*5*), which resemble tetrahedral intermediates in direct water attack. Recent experiments have shown that these inhibitors are bound noncovalently to the enzyme, their affinity based partly on hydrogen bonds resembling those believed to arise during general base catalysis (*34*). Although acid catalysis of adenosine deamination was observed in

this study, acid catalysis by the enzyme appears unlikely because deoxycoformycin is bound as the neutral form (*34*), and because the enzyme is active at high pH.

It is of interest to compare the present rate constants of uncatalyzed nucleoside deamination with the catalytic rate constants observed for adenosine and cytidine deaminases. The rate of uncatalyzed deamination of cytidine is 8.8×10^{-8} s^{-1} at 85°C. Using the relationship between rate and temperature shown in Fig. 1, the extrapolated rate would be 3.2×10^{-10} s^{-1} at 25°C. The data of Ashley and Bartlett (*12*) suggest a k_{cat} value of 137 s^{-1} and a k_{cat}/K_m of 7.5×10^5 M^{-1} s^{-1} for cytidine deaminase from *E. coli*, indicating that this enzyme is able to enhance the rate of reaction by a factor of 4×10^{11}; division of the rate enhancement by the K_m yields a value for K_{TX} of 4×10^{-16} M. For adenosine deamination, if it is assumed that the energy of activation for the uncatalyzed rate is the same as that for the hydroxide ion-catalyzed reaction, as was the case for cytidine deamination, then a similar calculation can be performed. Using 1.8×10^{-10} s^{-1} for the spontaneous, nonenzymatic, rate at 37°C (extrapolated from the spontaneous rate of 8.6×10^{-9} s^{-1} at 85°C), a k_{cat} of 375 s^{-1}, and a k_{cat}/K_m of 1.4×10^7 M^{-1} s^{-1} at 37°C (*35*), one obtains a rate enhancement of 2×10^{12}-fold and a transition state dissociation constant of 8×10^{-17} M. 2′-Deoxycoformycin, a inhibitor of adenosine deaminase with a K_i value of 2.5×10^{-12} M, is bound 10^7-fold more tightly than the substrate adenosine. Although this compound is one of the most potent transition state analog inhibitors known, it falls almost 10^5-fold short of the minimal affinity that would be expected of an ideal transition state of this reaction. This shortfall may not be surprising, considering the differences in structure between the unstable ideal and the stable reality, and the many differences in bonding details that would be expected to result.

The present results, along with minimal transition state affinities estimated for several other enzymes, are presented in Table 2. Although the sample size is small, deaminases appear to have relatively high affinities for their transition

TABLE 2

MINIMAL AFFINITIES OF ENZYMES FOR TRANSITION STATES

Enzyme	k_{non} (s^{-1})	k_{cat}/K_m[a] (M^{-1} s^{-1})	K_{TX} (M)	Ref.
Alkaline phosphatase	1.0×10^{-15}[b]	5.7×10^3	2×10^{-19}	(*36, 37*)
Acetylcholinesterase	1.1×10^{-8}[c]	1.6×10^8	7×10^{-17}	(*38, 39*)
Adenosine deaminase	1.8×10^{-10}	1.4×10^7	8×10^{-17}	(*35*)
Deoxycytidylate deaminase	9.2×10^{-11}	1.5×10^6	6×10^{-17}	(*29*)
Cytidine deaminase	3.2×10^{-10}	7.5×10^5	4×10^{-16}	(*12*)
Urease	3.0×10^{-10}	2.3×10^5	1×10^{-15}	(*40, 41*)
Triosephosphate isomerase	4.3×10^{-6}	9.1×10^6	5×10^{-13}	(*42, 43*)
Chorismate mutase	2.6×10^{-5}	1.1×10^6	2×10^{-11}	(*44–46*)
Carbonic anhydrase	3.7×10^{-2}	1.2×10^8	3×10^{-11}	(*47, 48*)

[a] In the calculation of k_{cat}, it was assumed that all subunits were equally active.

[b] Estimated by extrapolation to neutral pH.

[c] From the rate of ethyl acetate hydrolysis.

states. Values of k_{non} span 12.5 orders of magnitude, whereas values for k_{cat}/K_m span only 4.5 orders of magnitude. Thus, enzyme that catalyze slow reactions achieve the largest reductions in K_{TX}.

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