On the Rate-Determining Step in the Action of Adenosine Deaminase*

Richard Wolfenden

ABSTRACT: Takadiastase adenosine deaminase and calf duodenal adenosine deaminase show no appreciable primary deuterium isotope effect on the kinetics of hydrolysis of adenosine. The product inosine is a competitive inhibitor of both enzymes. The alternate product inosine monophosphate is a competitive inhibitor of the enzyme from takadiastase. The product ammonia is a noncompetitive inhibitor of the enzyme from takadiastase, with $K_{i\,app} = 3.9 \times 10^{-2}$ M for ammonia as the free base. Ammonia at concentrations as high as 1 M gives no appreciable inhibition of the enzyme from calf duodenum. These results, in conjunction with earlier data, support a mechanism involving rate-limiting isomerization of the enzyme–substrate complex and are consistent with rate-limiting formation of a tetrahedral intermediate.

Like many proteolytic enzymes, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) catalyzes the hydrolytic displacement of amines and other leaving groups from a type of electron-deficient carbon atom at which nucleophilic substitution reactions occur fairly readily in aqueous solution (Walsh and Wolfenden, 1967).

Earlier studies on takadiastase adenosine deaminase showed that a variety of leaving groups are removed from purine ribonucleosides at similar limiting rates (Wolfenden, 1966). This paper describes an investigation of deuterium isotope effects and product inhibition patterns which was undertaken in order to provide further information concerning the nature of the rate-limiting step in catalysis of adenosine deamination. Parallel studies were made on the enzyme from calf intestine, which catalyzes hydrolysis of a similar variety of leaving groups with different limiting rate constants (Cory and Suhadolnik, 1965; Chassy and Suhadolnik, 1967; Baer *et al.*, 1968).

Experimental Procedures

Adenosine deaminase from takadiastase was prepared as described previously (Wolfenden *et al.*, 1968). Adenosine deaminase from calf intestinal mucosa was obtained from Boehringer Mannheim Corp. Deuterium oxide (99.7%) was obtained from Merck Sharpe and Dohme of Canada, Ltd., and redistilled before use. Nucleosides and nucleotides were obtained from P-L Laboratories, Inc.

Kinetic measurements were made at 25° as described previously (Wolfenden *et al.*, 1967), using cuvets of 1-mm and 1-cm light paths. Rates observed at 260 mμ were recalculated in terms of concentration using the molar extinction coefficients of adenosine and inosine at that wavelength (Beaven *et al.*, 1955).

Results

Deuterium Oxide Effect. At pH 6.5 the substrate dependence of the rate of deamination of adenosine was measured in water and in 90% deuterium oxide (Table I). In this pH region, takadiastase adenosine deaminase does not show appreciable variation in K_m or V_{max} with pH (Wolfenden *et al.*, 1967). Table I shows that isotope effects on V_{max} and K_m were negligible for the enzyme from takadiastase.

Calf intestinal adenosine deaminase showed a decrease in V_{max} and K_m in D_2O, but virtually no effect on V_{max}/K_m. In this region V_{max}/K_m is insensitive to pH, but V_{max} and K_m of the calf intestinal enzyme show inflections (Baer *et al.*, 1968). It is therefore reasonable to suppose that D_2O effects on the isolated kinetic parameters may reflect changes in pK_a values of the enzyme–substrate complex.

Inhibition by Products. Takadiastase adenosine deaminase was inhibited competitively by the product inosine (Figure 1) with $K_i = 1.82 \times 10^{-3}$ M. Deamination of adenosine was also inhibited by IMP (the product of deamination of the alternate substrate 5′-AMP) with $K_i = 5.65 \times 10^{-4}$ M. These inhibition constants were measured at pH 4.7 in order that IMP should be mainly in its monanionic form. Inhibition by IMP disappeared at high pH values; it was previously demonstrated (Wolfenden *et al.*, 1967) that mononucleotides are effectively bound as substrates only in their monoanionic form.

Inhibition of takadiastase adenosine deaminase by ammonium ion at pH 6.5 could not be detected (Wolfenden, 1966). However substantial inhibition of takadiastase adenosine deaminase was observed at high pH values where appreciable concentrations of ammonia as free base are present. Figure 2 shows the effect of ammonium chloride buffers on the substrate dependence of adenosine deamination at pH 8.87. Ammonia buffer was found to be a noncompetitive inhibitor of adenosine deamination, with $K_{i\,app} = 1.4 \times 10^{-1}$ or 3.9×10^{-2} M in terms of the free-base ammonia.

Calf intestinal adenosine deaminase (which does not deam-

* From the Program in Biochemical Sciences, Frick Chemical Laboratory, Princeton University, Princeton, New Jersey 08540. *Received January 22, 1969.* Supported by Grant No. GM-12725 from the National Institutes of Health, U. S. Public Health Service.

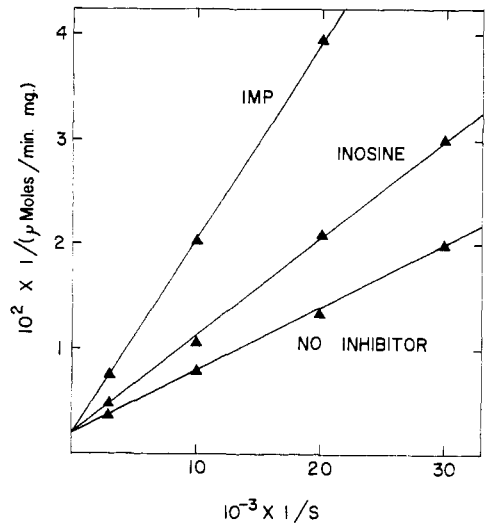


FIGURE 1: Double-reciprocal plot of the substrate dependence of the rate of deamination of adenosine by takadiastase adenosine deaminase in the absence of inhibitors and in the presence of inhibitory inosine and 5′-IMP, each at a concentration of 1.0×10^{-3} M. Rates were measured at 25° in 0.1 M potassium acetate buffer (pH 4.60).

inate AMP) was also competitively inhibited by inosine. At pH 6.50 (0.1 M potassium phosphate buffer), K_i for inosine was 1.6×10^{-4} M. The calf enzyme was not detectably inhibited by ammonium chloride buffers at concentrations as high as 2 M at pH 9.2.

Discussion

Three possibilities could be reconciled with the earlier observation of similar limiting rate constants for a variety of leaving groups from 6-substituted purine ribonucleosides (Wolfenden, 1966). (1) The bond between purine and leaving groups could be broken before the rate-limiting step occurred. (2) Isomerization of the free enzyme might be rate limiting.

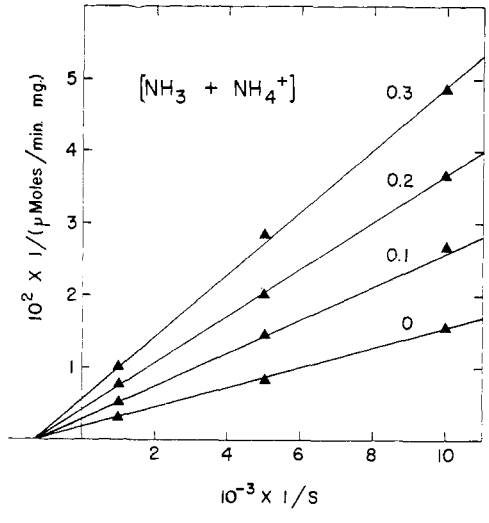


FIGURE 2: Double-reciprocal plot of the substrate dependence of the rate of deamination of adenosine by takadiastase adenosine deaminase in the presence of ammonia buffers (pH 8.87) at the concentrations indicated. Rates were measured at 25° in the presence of 1.0 M Tris-Cl buffer (pH 8.85).

TABLE I: Relative Activity of Adenosine Deaminases in Deuterium Oxide, at pH 6.50 (0.1 M Potassium Phosphate) at 25°.

	V_{max} (μmoles/min mg)	K_m (M)	V_{max}/K_m
Takadiastase			
H_2O	510	2.4×10^{-4}	2.1×10^6
90% D_2O	517	2.6×10^{-4}	2.0×70^6
(H/D)	(0.98)	(0.92)	(1.05)
Calf duodenum			
H_2O	217	3.3×10^{-5}	6.5×10^6
90% D_2O	153	2.5×10^{-5}	6.1×10^6
(H/D)	(1.42)	(1.33)	(1.07)

(3) The bond between purine and leaving groups might not be stretched appreciably during passage from the Michaelis complex to the transition state for the over-all reaction. Isomerization of the enzyme–substrate complex would then be rate limiting.

The first possibility, cleavage of the leaving group before the transition state, appears unlikely in view of the absence of a primary deuterium isotope effect. The absence of a primary deuterium isotope effect for adenosine deaminase contrasts with the behavior of several proteolytic enzymes which show $k_H/k_D = 2.6$–3.6 (Mason and Ghiron, 1961; Bender and Hamilton, 1962; Caplow and Jencks, 1962; Bender and Brubacher, 1964), and suggests that the substrate water is not appreciably stretched in the transition state for the reaction catalyzed by adenosine deaminase. If hydrolysis of a common purinyl–enzyme intermediate were rate limiting, water would be at least partly cleaved in the transition state. If release of the product inosine were rate limiting, water would be cleaved before the rate-limiting step. Rate-limiting isomerization of a purinyl–enzyme intermediate could occur, with cleavage of the leaving group before the transition state and entry of water after the transition state. However there appears to be a more serious objection to the departure of leaving groups before the transition state which will now be discussed.

For reactions proceeding through a common transition state R-enz$^{\mp}$, with prior release of the variable leaving group X, variations in the nature of X would be expected to produce parallel effects (a) on the over-all equilibrium constant for hydrolysis of R–X, and (b) on the rate constant, V_{max}/K_m, for attainment of the transition state for the enzyme-catalyzed reaction. This follows if rate constants are regarded as proportional to equilibrium constants for attainment of the transition state (Glasstone *et al.*, 1941), and from the fact that R–X and H–X are common variables in eq a and b, when reactions are measured under the same conditions.

$$R\text{-}X + H_2O \rightleftharpoons R\text{-}OH + H\text{-}X \qquad (a)$$

$$R\text{-}X + H\text{-}enz \rightleftharpoons R\text{-}enz^{\mp} + H\text{-}X \qquad (b)$$

Free energies of hydrolysis of 6-substituted purine ribonucleosides have not been determined in neutral solution ex-

TABLE II: Approximate Contributions to Free Energy of Hydrolysis of 6-X Purine Ribonucleosides.

	E(C–X) − E(H–X) (kcal/mole)	ΔF Ionization of HX (at pH 6.5) (kcal/mole)	Sum (kcal/mole)
Cl	−22	−17.8[a]	−40
OCH_3	−25.1	0	−25
NH_2	−20.6	−3.8	−24

[a] Based on pK(HCl) = −6 at 25° (Robinson, 1936).

cept in the case of adenosine (Wolfenden *et al.*, 1967); however, relative values for hydrolysis of different leaving groups may be very roughly estimated from their relative bond energies to carbon and hydrogen (Cottrell, 1960) and from the free energy of ionization of their conjugate acids in buffered solution near neutrality. This calculation ignores possible effects of aromaticity and solvation, but should serve as a crude indication of the expected effect. The free energy of hydrolysis of 6-chloropurine ribonucleoside should be at least 15 kcal/mole more negative than that for adenosine and for 6-methoxypurine ribonucleoside (Table II). In fact the activation energy for enzyme-catalyzed hydrolysis (as indicated by V_{max}/K_m values for takadiastase adenosine deaminase) is lower for adenosine than for 6-chloropurine or 6-methoxypurine ribonucleosides, which are comparable (Table III). In view of these considerations, the similar V_{max} values for diverse leaving groups suggest that little or no stretching of the bond from purine to leaving group occurs as reactions catalyzed by takadiastase adenosine deaminase proceed from the Michaelis complex to the transition state.

Limiting rates of hydrolysis of different leaving groups by other enzymes of this type are not so closely similar as in the case of takadiastase adenosine deaminase. It is however worth noting that V_{max} values fall within a rather narrow range for calf intestinal adenosine deaminase working on 6-substituted nitrogen, oxygen, and chloro derivatives of purine ribonucleoside and 2-aminopurine ribonucleoside (Chassy and Suhadolnik, 1967; Baer *et al.*, 1968) and for the adenases of *Azotobacter* and *Candida utilis* working on nitrogen and chloro derivatives of purine (Hartenstein and Fridovich, 1967) (Table III). In these instances V_{max} values fall within one order of magnitude of each other.

The second possibility considered above, rate-limiting isomerization of free enzyme, is rendered unlikely by the results of product inhibition experiments with takadiastase adenosine deaminase. Competitive inhibition by inosine (Figure 1) and noncompetitive inhibition by ammonia (Figure 2) are consistent with the simple Uni-Bi mechanism indicated below (which does not specify the point of water entry), and not with any ordered mechanism in which slow isomerization of the free enzyme occurs after inosine release and before binding of adenosine (Cleland, 1963).

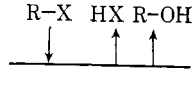


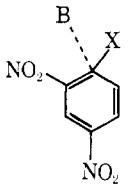
TABLE III: Relative Rates of Enzymatic Deamination of 6-Substituted Purine Derivatives.

	X	Rel V_{max}	V_{max}/K_m
Takadiastase adenosine deaminase			
+ 6-X-Pu-R	NH_2	1.0	4,000[a]
	Cl	1.16	23[a]
	OCH_3	1.09	7[a]
Calf duodenal adenosine deaminase			
+ 6-X-Pu-R	NH_2	1.0	20,000[b]
		1.0	34,000[c]
	Cl	0.23	320[b]
		0.21	840[c]
+ 6-X-2-NH_2-Pu-R	NH_2	0.25	7,400[c]
	Cl	0.73	1,100[b]
		1.27	4,200[c]
Azotobacter adenase			
+ 6-X-Pu	NH_2	1.0	100,000[d]
	Cl	0.62	1,200[d]
			570[d]
Candida utilis adenase			
+ 6-X-Pu	NH_2	1.0	33,300[d]
	Cl	0.29	116[d]

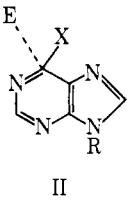
[a] Wolfenden (1966). [b] Chassy and Suhadolnik (1967). [c] Baer *et al.* (1968). [d] Hartenstein and Fridovich (1967).

If neither water nor the susceptible bond in the substrate is cleaved as the reaction proceeds from the Michaelis complex to the transition state, consideration must be given to the third possibility, rate-limiting isomerization of the enzyme–substrate complex before bond cleavage occurs. Possible slow steps would then be (a) a conformational change of the enzyme which is not sensitive to the nature of 6-substituents on the substrate, or (b) rate-limiting nucleophilic attack by the enzyme (or water bound to the enzyme) on the substrate without appreciable stretching of the bond to leaving group. Chemical analogies suggest that b should be given serious consideration, especially in view of the effectiveness of 6-chloropurine ribonucleoside as an alkylating agent (Walsh and Wolfenden, 1967). When formation of a tetrahedral intermediate constitutes the rate-determing step in the over-all reaction, nucleophilic substitution reactions are frequently insensitive to the nature of the leaving group. Thus piperidine (Bunnett *et al.*, 1957) and thiophenoxide (Bunnett and Merritt, 1957) react with a striking variety of 1-substituted 2,4-dinitrobenzenes at virtually identical rates, indicating that the bond to leaving groups is not stretched appreciably in the transition state for nucleophilic substitution (I). In nucleophilic substitutions in aqueous solution, formation of a tetrahedral intermediate appears to be rate determining in reactions of both purine and dinitrobenzene derivatives (Walsh and Wolfenden, 1967). It is therefore reasonable to speculate that the transition state in reactions of adenosine deaminase may be represented as incip-

ient formation of a tetrahedral intermediate involving enzyme (or enzyme-bound water) and substrate (II).



I



II

In the case of calf intestinal adenosine deaminase, where there is variation of V_{max} with leaving group, it appears likely that stretching of the bond to leaving group may occur in the inversion in order of mobility of ammonia and chloride as between 6-substituted purine ribonucleosides and 6-substituted 2-aminopurine ribonucleosides (Table III) illustrates the subtle balance between bond making and bond breaking which may apply in this case. However there are substrates of the mammalian enzyme for which V_{max} values are very much lower than that for adenosine (Chassy and Suhadolnik, 1967; Baer *et al.*, 1968) and it apears likely that bond breaking dominates the kinetic behavior of these substrates.

References

Baer, H. P., Drummond, G. I., and Gillis, J. (1968), *Arch. Biochem. Biophys. 123*, 172.

Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), *in* The Nucleic Acids, Chargaff, E., and Davidson, J. N., Ed., New York, N. Y., Academic, p 493.

Bender, M. L., and Brubacher, L. J. (1964), *J. Am. Chem. Soc. 86*, 5333.

Bender, M. L., and Hamilton, G. A. (1962), *J. Am. Chem. Soc. 84*, 2570.

Bunnett, J. F., Garbisch, E. W., and Pruitt, K. M. (1957), *J. Am. Chem. Soc. 79*, 385.

Bunnett, J. F., and Merritt, W. D. (1957), *J. Am. Chem. Soc. 79*, 5967.

Caplow, M., and Jencks, W. P. (1962), *Biochemistry 1*, 883.

Chassy, B. M., and Suhadolnik, R. J. (1967), *J. Biol. Chem. 242*, 3655.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 188.

Cory, J. G., and Suhadolnik, R. J. (1965), *Biochemistry 4*, 1753.

Cottrell, T. L. (1960), The Strengths of Chemical Bonds, Ithaca, N. Y., Cornell, p 270.

Glasstone, S., Laidler, K. J., and Eyring, H. (1941), Theory of Rate Processes, New York, N. Y., McGraw-Hill.

Hartenstein, R. C., and Fridovich, I. (1967), *J. Biol. Chem. 242*, 740.

Mason, R., and Ghiron, C. A. (1961), *Biochim. Biophys. Acta 51*, 377.

Robinson, R. A. (1936), *Trans. Faraday Soc. 32*, 743.

Walsh, B. T., and Wolfenden, R. (1967), *J. Am. Chem. Soc. 89*, 6221.

Wolfenden, R. (1966), *J. Am. Chem. Soc. 88*, 3157.

Wolfenden, R., Sharpless, T. K., and Allan, R. (1967), *J. Biol. Chem. 242*, 977.

Wolfenden, R., Tomozawa, Y., and Bamman, B. (1968), *Biochemistry 7*, 3965.

Ring-Modified Substrates of Adenosine Deaminases*

R. Wolfenden, J. Kaufman, and J. B. Macon

ABSTRACT: Adenosine deaminases (adenosine aminohydrolase, EC 3.5.4.4) from the mould *Aspergillus oryzae* and from calf intestine catalyze deamination of 4-aminopteridine, 1-methyladenosine, 3-β-(D-ribofuranosyl)adenine, and unsubstituted adenine. Unsubstituted pteridine is an effective inhibitor of both enzymes.

When adenine derivatives are compared, electron-withdrawing groups are found to increase the limiting rate of enzymatic deamination. The presence of ribose as a substituent on either the 3 or the 9 position, or the replacement of the 8-carbon by sulfur or oxygen, result in large increases in V_{max} as compared with adenine. Similarly, 8-trifluoromethylpurine is a much more effective inhibitor than unsubstituted purine.

Adenosine deaminases (adenosine aminohydrolase, EC 3.5.4.4) have been isolated as pure proteins from calf intestine (Brady and O'Connell, 1962) and from *Aspergillus oryzae* (takadiastase) (Wolfenden *et al.*, 1968). Both enzymes appear to be protein catalysts not requiring cofactors. Both exhibit considerable nonspecificity. The enzyme from takadiastase rapidly deaminates adenine nucleosides, nucleotides, and oligonucleotides (Wolfenden *et al.*, 1967). Both enzymes catalyze hydrolytic cleavage of nitrogen-, halogen-, oxygen-, and sulfur-leaving groups from 6-substituted purine ribonucleosides (Cory and Suhadolnik, 1965; Wolfenden, 1966; Baer and Drummond, 1966; Wolfenden and Kirsch, 1968).

This paper explores another aspect of the catalytic versatility

* From the Program in Biochemical Sciences, Frick Chemical Laboratory, Princeton University, Princeton, New Jersey 08540. *Received January 22, 1969.* Supported by Research Grant No. GM-12725 from the National Institutes of Health, U. S. Public Health Service.