Dechloronase Activity of Adenosine Deaminase*

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ABSTRACT: Adenosine deaminase catalyzes the dechlorination of 6-chloropurine ribonucleoside yielding as products of the reaction inosine and chloride ions in stoichiometric amounts. The Michaelis constant for the 6-chloropurine ribonucleoside is 6.4×10^{-4} M as compared with 8.3×10^{-5} M for adenosine. The V_m for the dechlorination reaction is 0.25 that of the deamination reaction. Using 6-chloropurine ribonucleoside as the substrate, the enzymatic dehalogenation is inhibited when adenosine is added to the reaction mixture. When 6-chloropurine ribonucleoside is added to the enzyme reaction mixture containing adenosine as the substrate, the deamination reaction is inhibited. Compounds which inhibit the deamination reaction also inhibit the dechlorination reaction. These data suggest that the same active site on the deaminase is involved in both the deamination and the dehalogenation reactions.

In a study of the structural requirements of compounds that can react at the active site of adenosine deaminase, it was observed that this enzyme could also convert 6-chloropurine ribonucleoside to inosine. In addition to this report there have been several other publications concerned with enzymatic dehalogenations. Enzymatic defluorination of *p*-fluorophenylalanine to form tyrosine by the phenylalanine-hydroxylating system has been shown to occur in rat liver preparations (Kaufman, 1961). In this reaction the fluoride is replaced by a hydroxyl group. More recently, Kearney *et al.* (1964) also reported on the enzymatic dechlorination of 2,2′-dichloropropionic acid. The final product was pyruvic acid. In this paper we report on the nature of the dechloronase reaction as catalyzed by adenosine deaminase.

Materials and Methods

Adenosine deaminase (calf intestinal mucosa, type I, 210 units/mg) and 6-chloropurine ribonucleoside were purchased from the Sigma Chemical Co. The dechlorination reaction was measured by following either the rate of disappearance of 6-chloropurine ribonucleoside at 265 mμ or the rate of appearance of inosine at 250 mμ. The assay in which inosine formation was measured was superior to the assay in which the disappearance of 6-chloropurine ribonucleoside was measured. The change in molar absorbance at 250 mμ is 5.6×10^3 M^{-1} cm^{-1} and the change in molar absorbance at 265 mμ is -4.0×10^3 M^{-1} cm^{-1}. Therefore, product formation was generally used to assay the reaction. Assay mixtures contained substrate, sodium phosphate buffer, *p*H 7.0, 0.05 M, and 0.084 unit of adenosine deaminase in a final volume of 1.5 ml. The rates of reaction were measured on a Beckman DU spectrophotometer at 24–26° in cells of 5-mm light path length.

Inosine was measured as the product of the reaction by paper chromatography using butanol–2% NH_3 (86:14) and ammonium sulfate–sodium phosphate buffer–1-propanol (60 g:100 ml, *p*H 7.0, 0.1 M:2 ml) as the developing solvents.

Chloride was determined quantitatively by the coulometric titration with Ag^+, using an Aminco-Cotlove chloride titrator. Qualitative measurements were made by using the mercuric chloroanilate procedure (Hodge and Gerarde, 1963).

Results

Stoichiometry of Reaction. Dechlorination of 6-chloropurine ribonucleoside, in the presence of adenosine deaminase, gives inosine and chloride ions as the products. The rate of disappearance of 6-chloropurine ribonucleoside is in quantitative agreement with the rate of inosine formation. The stoichiometry of the reaction products is such that 1 mole of chloride ions is produced per mole of inosine (Table I).

Kinetic Parameters of 6-Chloropurine Ribonucleoside and Adenosine. In Table II the kinetic parameters K_m and V_m are compared for adenosine and 6-chloropurine ribonucleoside. The Michaelis constant for 6-chloropurine ribonucleoside is approximately one order of magnitude greater than that for adenosine, while the V_m is only one-fourth as large as that of adenosine.

*p*H *Optimum.* The *p*H curve for 6-chloropurine ribonucleoside is shown in Figure 1 and is very similar to that for adenosine, although the initial rates are much lower for 6-chloropurine ribonucleoside. This

* From the Research Laboratories, Department of Biochemistry, Albert Einstein Medical Center, Philadelphia, Pa. *Received October 26, 1964; revised June 21, 1965.* This investigation was supported by a grant (GM08685-04) from the National Institutes of Health and a grant (G21239) from the National Science Foundation.

† Research Career Development Awardee of the National Institutes of Health (5-K3-GM-7100-04).

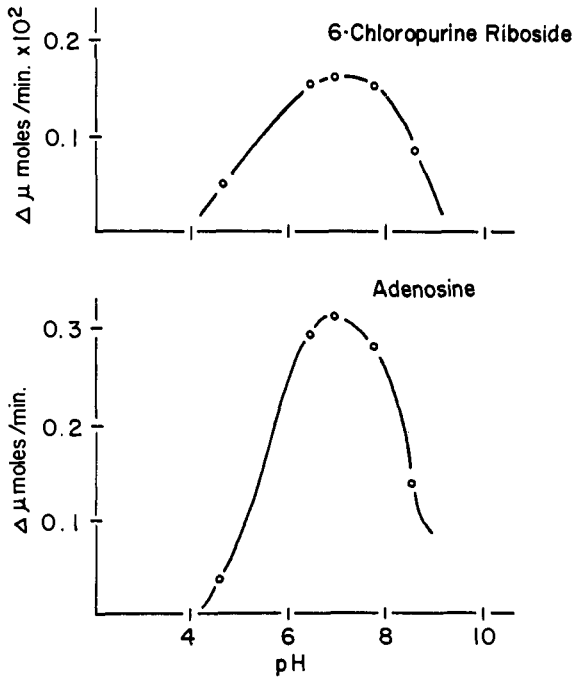

FIGURE 1: Comparison of *p*H versus concentration curves of 6-chloropurine riboside and adenosine. Substrate concentration 5.3×10^{-5} M, sodium phosphate buffer 0.05 M, and 0.084 unit of adenosine deaminase, final volume 1.5 ml.

TABLE I: Stoichiometry of the Reaction Products.[a]

	Inosine (μmoles/hr)	Cl^- (μmoles/hr)	Cl^-/ Inosine
Reaction mixtures[b]	0.48	0.43	0.9

[a] Reaction mixtures contained substrate 5.3×10^{-4} M; sodium phosphate buffer, *p*H 7.0, 0.05 M; and 0.084 unit of adenosine deaminase in a final volume of 1.5 ml. Inosine formation followed at 250 mμ. After 1 hour, 0.1 ml. of 5 N HNO_3 was added to stop the reaction. Chloride ion concentration was then determined. [b] These values are the average of six identical determinations.

*p*H optimum is consistent with that reported by Kalckar (1947).

Effect of Deaminase Inhibitors. Compounds which have been shown to competitively inhibit adenosine deamination by adenosine deaminase (Cory and Suhadolnik, 1965) also inhibit the dechlorination reaction. These results are shown in Table III. There are no exceptions. The degree of inhibition of those inhibitors for the 6-chloropurine ribonucleoside is relatively the same as for adenosine.

Effect of Adenosine on Dechlorination Reaction. In the presence of adenosine (5×10^{-5} M) the dechlorination of 6-chloropurine ribonucleoside (6.4×10^{-4} M) was inhibited 75%. This reaction was measured at 250 mμ.

TABLE II: Kinetic Constants for Adenosine Deaminase.

Substrate	K_m (M)	V_m[a]
Adenosine	8.3×10^{-5}	400
6-Chloropurine ribonucleoside	6.4×10^{-4}	100

[a] μmoles/min/mg protein.

TABLE III: Effect of Deaminase Inhibitors on Dechlorination Reaction.[a]

Inhibitor	Inhibition (%)
N^6-Methyladenosine	94
N^6-Methyldeoxyadenosine[b]	48
9-(*n*-Pentyl)adenine[b]	55
9-(*n*-Hexyl)adenine[b]	70
6-Mercaptopurine ribonucleoside	25
9-Purine ribonucleoside	87

[a] Reaction mixtures contained final concentrations 2.66×10^{-4} M substrate, 5.3×10^{-5} M inhibitor, and 0.084 unit of adenosine deaminase. [b] Gifts from Drs. J. Fox and J. Montgomery.

Effect on 6-Chloropurine Ribonucleoside on Adenosine Deamination. In the presence of 6-chloropurine ribonucleoside (3.2×10^{-4} M) the deamination of adenosine (5×10^{-5} M) was only slightly inhibited (12%), as would be expected, since the K_m of adenosine is 8.3×10^{-5} M while the K_m of 6-chloropurine ribonucleoside is 6.4×10^{-4} M. This reaction was measured at 265 mμ.

Reversibility of Reaction. In the presence of inosine, chloride ions, and adenosine deaminase no 6-chloropurine ribonucleoside is formed.

Discussion

It has been shown that 6-chloropurine ribonucleoside is a substrate for the enzyme, adenosine deaminase. The products of the reaction are inosine and chloride ions which are formed in stoichiometric amounts.

According to Dixon and Webb (1958), for a single enzyme catalyzing two separate reactions the total velocity of the reactions must lie between the two velocities which would be obtained if the reactions were carried out separately. In the presence of adenosine, the rate of the dechlorination reaction is inhibited, while in the presence of 6-chloropurine ribonucleoside, the rate of the deamination reaction is slightly inhibited.

This is what would be expected on the basis of the Michaelis constants of the respective substrates.

In addition, the same compounds which are competitive inhibitors of the deamination reaction (Cory and Suhadolnik, 1965) are also inhibitors of the dechlorination reactions. Presumably this inhibition is also of the competitive type.[1] The dechlorination reaction was shown not to be reversible. This result is in agreement with the data reported by Kalckar (1947) on the irreversibility of the deamination reaction.

It is concluded that the same enzyme and the same active site on adenosine deaminase is involved in both the dechlorination and deamination reactions. That 6-chloropurine ribonucleoside can be converted to inosine and Cl^- by adenosine deaminase could complicate studies involving the use of this compound as an anticancer agent.

[1] The nature of the assay system for the dechlorination of 6-chloropurine ribonucleoside, that is, the change in absorbance at 250 and 265 mμ, the relatively high K_m for 6-chloropurine ribonucleoside, and the high extinction coefficients of both the substrates and inhibitors at these wavelengths, makes it technically difficult to determine the type of inhibition imposed on the dechlorination reaction.

Acknowledgment

The authors gratefully acknowledge Dr. Louis Delavan of Women's Medical College of Pennsylvania for the chloride coulometric titrations.

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The Primary Specificity of α-Chymotrypsin. Interaction with Acylated Derivatives of D-Valine Methyl Ester and D-Norvaline Methyl Ester*

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ABSTRACT: The chymotrypsin-catalyzed hydrolysis of acetyl-L-leucine methyl ester is inhibited by derivatives of acylated D-valine and D-norvaline methyl esters. Competitive inhibition constants for the D-valine derivatives are acetyl $> 195 \times 10^{-3}$ M, chloroacetyl $= 169 \times 10^{-3}$ M, and benzoyl $= 5.2 \times 10^{-3}$ M. For the corresponding D-norvaline compounds the values are 111×10^{-3} M, 58.6×10^{-3} M, and 1.77×10^{-3} M, respectively. The chymotrypsin-catalyzed hydrolysis of *N*-benzoyl-L-norvaline methyl ester follows Michaelis-Menten kinetics with $K_0 = 0.85 \pm 0.16 \times 10^{-3}$ M and $k_0 = 2.45 \pm 0.14$ sec^{-1}. The values of the ratios of binding constants of the L and D enantiomers [K_0(L)/K_0(D)] support earlier conclusions that the acetyl- and chloroacetyl-L-valine and -L-norvaline methyl esters are bound predominantly in a productive mode, whereas the corresponding benzoyl derivatives of valine are mainly bound in a nonproductive manner. The data for the benzoyl norvaline methyl esters suggest that these binding constants are also controlled by more than one mode of combination. As observed previously in the L series, the isopropyl side chain of the D-valine esters offers greater steric hindrance to binding than does the *n*-propyl group of the D-norvaline compounds.

The primary specificity of chymotrypsin, that is, its catalytic behavior toward compounds containing only a single amino acid residue or related structures, has been the subject of intensive study during the past 15 years (Niemann, 1964). A general correlation between substrate and enzyme specificity has been developed (Hein and Niemann, 1961, 1962) and has since been applied successfully to explain the catalytic

* From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena. Contribution No. 3185. *Received November 12, 1964; revised June 21, 1965.* Supported in part by a grant from the National Institutes of Health, U.S. Public Health Service.

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