ON THE MECHANISM OF ADENOSINE DEAMINASE ACTION*

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Adenosine deaminase (Adenosine aminohydrolase, EC 3.5.4.4) from calf intestinal mucosa and from rat heart muscle was found to hydrolyze 6-chloropurine ribonucleoside (Cory and Suhadolnik, 1965a) and 2-amino-6-chloropurine ribonucleoside (Bär et al., 1966) with formation of inosine and guanosine, respectively. From these observations, it seemed that the basic mechanism of action was not a specific aminohydrolysis but rather appeared to constitute the catalysis of a nucleophilic displacement by hydroxyl at the 6-position of the purine system. To further test this hypothesis, additional 6-substituted derivatives of purine ribonucleoside (PRN) were used. It was demonstrated that the enzyme from intestinal mucosa was, indeed, capable of hydrolyzing other substituents including methylamino-, hydroxylamino- and methoxy- groups. Structural considerations of the substrates make it apparent that the broad specificity of the enzyme is in analogy to amide and ester splitting enzymes.

MATERIALS AND METHODS

Adenosine deaminase from calf intestinal mucosa was purchased from Sigma Chemical Company and dialyzed against distilled H_2O prior to use. Possible substrates were tested by incubating 0.1 µmole of substance in 2 ml 0.05 M Tris-HCl buffer, pH 7.5, at room temperature with 0.7 units[1]

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[1]One unit is the amount of enzyme which deaminates 1 µmole of adenosine per minute at 30°, as determined with a substrate concentration of 0.67×10^{-4} M in 0.05 M potassium phosphate buffer, pH 6.8.

of the enzyme. Recording of UV-spectra was performed before and after the addition of enzyme at suitable time intervals, using a Bausch and Lomb Spectronic 505 instrument. K_m and K_I values were calculated from double reciprocal plots, and rate values were determined optically on a Beckman DU-instrument.

RESULTS AND DISCUSSION

Substrates of adenosine deaminase are listed in Table I.

TABLE I: Substrates of Adenosine Deaminase

Substrate	Product	λmax pH 7.5 Substrate	λmax pH 7.5 Product	Relative rates of hydrolysis*
Adenosine	inosine	259	249	100
6-Hydroxylamino-PRN	inosine	266	249	17
2-Amino-6-chloro-PRN	guanosine	307, 247	253	12
2-Amino-6-methoxy-PRN	guanosine	280, 248	253	9
6-Methoxy-PRN	inosine	250	249	0.40
N^6-Methyladenosine	inosine	267	249	0.25

*As determined in 0.05 M potassium phosphate buffer, pH 6.8, at substrate concentrations 0.5 x 10^{-4} M and 30°.

Although the enzyme was not pure, it is reasonable to assume that only one activity is involved. For example, N^6-methyladenosine is a competitive inhibitor of adenosine deamination (Cory and Suhadolnik, 1965b), which is evidence that the same enzyme and active site are involved in the hydrolysis of the former to inosine. Furthermore, purine ribonucleoside is a potent competitive inhibitor of the enzyme (Cory and Suhadolnik, 1965b), and we found similar inhibitor constants for different substrates, i.e. adenosine (K_I = 0.096 x 10^{-4} M), 2-aminoadenosine (K_I = 0.11 x 10^{-4} M), 6-hydroxylamino-PRN (K_I = 0.12 x 10^{-4} M) and 2-amino-6-methoxy-PRN (K_I = 0.077 x 10^{-4} M), which lends support to the idea that one enzyme is responsible.

The hydrolysis of 6-methoxy-PRN was accompanied by a slight spectral

shift of λmax from 250 to 249 mμ with a decrease of only 4% in intensity. Further characterization of the product appeared necessary, therefore, and was carried out as follows. The ratios of absorbancies of the product were 280/260 = 0.25 and 250/260 = 1.67 (inosine = 0.24 and 1.69) as compared to < 0.05 and 1.54 for 6-methoxy-PRN, and the spectral shifts after addition of small amounts of concentrated HCl and KOH were identical with those obtained with authentic inosine. Finally, the product co-chromatographed with inosine on thin layer chromatography (silica gel/acetone). When 2-amino-6-methoxy-PRN was tested, the alteration of the spectrum was more drastic, the product showing the characteristic spectrum of guanosine. No spectral shifts were observed when 6-mercapto- and 6-methylmercapto-PRN were incubated with adenosine deaminase.

The lack of specificity of the enzyme in hydrolyzing amino-, chloro- and methoxy-groups can be explained if the catalytic action is generalized as a nucleophilic displacement of the substituent at C^6 of PRN. The hydrolysis of N^6-methyladenosine and of 6-hydroxylamino-PRN are consistent with this view, although it must be remembered that they are close derivatives of adenosine and would still constitute an aminohydrolysis. The properties of the deaminase seem to be related to the specificity of some esterases and proteolytic enzymes which are capable of hydrolyzing esters and amides. This comparison appears valid if one considers the lactim form of inosine as the imido form of a carboxylic acid, so that the 6-chloro, 6-methoxy and 6-amino derivatives resemble an acid chloride, a methyl ester and an amide, respectively. Further support of this concept is the fact that we observed inhibition of the enzyme with diisopropylfluorophosphate (64% and 82% inhibition after 15 min and 45 min preincubation at room temperature with 10^{-3} M DFP) which suggests the possible action of an active serine group as is the case with many esterases and proteolytic enzymes. No inhibition occurred with iodoacetamide and N-ethylmaleimide, but p-chloromercuribenzoate caused inactivation which could be reversed in part with cysteine (Ronca et al., 1965). No evidence has been produced, though, as to the function of the particular thiol groups.

Since adenosine and 6-methoxy-PRN are hydrolyzed at considerably different

rates, the hydrolysis of an "acyl-enzyme"-like intermediate cannot be rate limiting as observed in the case of some proteolytic enzymes acting on esters and amides (Bender and Kézdy, 1965). Other 2-substituted adenosine derivatives like 2-amino- and 2-fluoro-adenosine have been reported as substrates of adenosine deaminase (Kornberg and Pricer, 1951; Chilson and Fisher, 1963), and we have demonstrated that 2-hydroxy- and 2-chloroadenosine are hydrolyzed with formation of xanthosine and 2-chloroinosine. The following order of reactivity was found with substrate concentrations of 10^{-4} M, when the 2-substituent was varied: $H \gg NH_2 > F > OH \gg Cl$. The effect of these substituents on the maximal rate of deamination will possibly reveal more details of the mechanism of hydrolysis. It is apparent that the major influence is not what would be expected from a nucleophilic displacement on aromatic systems, since electron withdrawing groups like chloro and fluoro should activate and increase the reactivity. The opposite effect would be expected, in fact, with respect to protonation of N^1 or N^3, a step which then would facilitate attack at the C^6 position.

In summary, adenosine deaminase might be considered as an amidase with regard to its natural substrate adenosine, since it exhibits a broad specificity for the group to be hydrolyzed. This hypothesis, involving the special case of a cyclic conjugated system, can be further explored using suitably substituted derivatives of purine ribonucleoside.

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