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AFFINITY LABELING OF HISTIDINE AND LYSINE RESIDUES IN THE ADENOSINE DEAMINASE SUBSTRATE BINDING SITE

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Summary

1. Adenosine deaminase was inactivated by 9-(4-bromoacetamidobenzyl)-adenine (I) and 9-(2-bromoacetamidobenzyl)adenine (II), two affinity labels.

2. The stoichiometry of the reaction with reagent II is reported: 1 mol reagent is bound per mol inactive enzyme. Amino acid analysis of the 6 N HCl hydrolyzate of the inactive enzyme identified CM-histidine as the main alkylation product. This is the first evidence of the presence of a histidine in the active site region.

3. The alkylation rate and involved amino acid residues were studied for both reagents I and II, at pH 8 and 5.5. The particular reactivity of a lysine near or in the active site is discussed.

Introduction

Adenosine deaminase plays a key role in metabolism of adenosine, a basic compound responsible for important pharmacological and toxic effects. A deficiency of this enzyme, in some cases of severe congenital immunodeficiency, represents the first link between immunological and enzymic defect [1].

Much research has been carried out [2—5] to define the contribution by purine and/or ribose moieties in binding a substrate, in order to find the effective inhibitors of the enzyme.

Utilizing as affinity label one of these inhibitors, that is 9-(4-bromoacetamidobenzyl)adenine (I), we pointed out [6] that the enzyme inactivation was related to alkylation of a lysine residue. Schaeffer et al. [5,7], who first

synthesized reagent I, prepared also several irreversible inhibitors capable of inactivating the same enzyme. Among those, 9-(2-bromoacetamidobenzyl)-adenine (II), which exhibited an affinity for the enzyme (K_i = 430 μM) unlike reagent I (K_i = 9 μM), alkylated the enzyme at a higher rate.

Schaeffer and Odin [7] pointed out that different residues might be involved in the alkylation. On this basis, taking advantage of a new quick method, we synthesized reagent II [8] in order to study the stoichiometry of the reaction between reagent II and the enzyme and to identify the alkylated amino acid(s). In the present paper these results are given. The pH effects in the alkylation of the enzyme with reagent I and II are also reported.

Materials and Methods

Adenosine deaminase from calf intestinal mucosa was purified by affinity chromatography [9] and showed a specific activity of 640 units/mg. The reagents I [10] and II [8] and the affinity adsorbent [9] were prepared as previously described; the CM-derivatives of lysine, histidine and methionine were prepared according to Gundlach et al. [11,12]; *S*-CM-cysteine was purchased from Schwarz/Mann (Biochemicals), Orangeburg, NY.

The concentration of the enzyme was measured by the microbiuret and Lowry methods, using freeze-dried enzyme as standard, or from its absorbance at 280 nm ($\epsilon = 31.8 \pm 0.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$) [6]. The molecular weight of adenosine deaminase was considered to be 35 000 [13,14]. The enzymic activity was determined with adenosine as substrate, according to Kalckar [15] in 0.1 M sodium phosphate buffer at pH 7. One enzyme unit is the amount of enzyme which will convert 1 μmol adenosine to inosine per min at 37° C and at pH 7. All activities given are the average of, at least, two assays.

Adenosine deaminase (0.5—100 μM) was treated in the dark at 37° C in 0.2 M sodium phosphate buffer (pH 8) or in 0.2 M sodium acetate buffer (pH 5.5), containing 10% (v/v) Me_2SO, with reagent I or II (0.1—2 mM) according to the method previously described [6].

The enzyme, treated with reagent II at various degrees of inactivation, was passed through a Sephadex G-25 (medium) column, according to the previous method described for reagent I [6]. The 100% inactive enzyme was obtained by passing partially inactivated (by reagent I or II) enzyme, after the Sephadex G-25 filtration through a 9-(4-aminobenzyl)adenine-Sepharose column (1.0 × 1.5 cm) (the affinity column) equilibrated with 0.1 M phosphate [10]. The equivalents of reagent I or II bound to protein were estimated spectrophotometrically, as previously extensively described for reagent I [6,10]. The method is based on the assumption that the spectrum of the alkylated enzyme results from the addition of enzyme and inhibitor spectra. At the isosbestic points, the sum of molar concentration of adenosine deaminase and the reagent bound to the enzyme is calculated. Ultraviolet spectra were determined with a Zeiss PMQ II spectrophotometer.

Protein hydrolysis was carried out in evacuated sealed vials according to the method of Moore and Stein [16] for the highest recovery of *S*-CM-cysteine and tyrosine. Amino acid analysis was performed on a Multichrome amino acid analyzer.

Results

Inactivation of adenosine deaminase by 9-(2-bromoacetamidobenzyl)adenine (II)

When adenosine deaminase was treated at pH 8 with reagent II, a rapid enzyme inactivation was observed, as shown in Fig. 1. Since the reagent was in excess with respect to the enzyme, a straight line was obtained on plotting log of percentage of residual adenosine deaminase activity against time. As previously reported [5,6], since the pseudo first-order constant of experimental rate was not proportional to concentration of the affinity labeling, it was possible to calculate the reaction rate constant (k_2). The k_2 value of reagent II at pH 8 resulted $7.6 \cdot 10^{-2}$ min^{-1}, which is in agreement with the value obtained by Schaeffer and Odin [7].

The competitive inhibitors of adenosine deaminase, like purine riboside, strongly protected against enzyme inactivation by reagent II (Fig. 2).

Stoichiometry of the reaction of adenosine deaminase with 9-(2-bromoacetamidobenzyl)adenine (II)

The stoichiometry of the reaction of adenosine deaminase with reagent I was

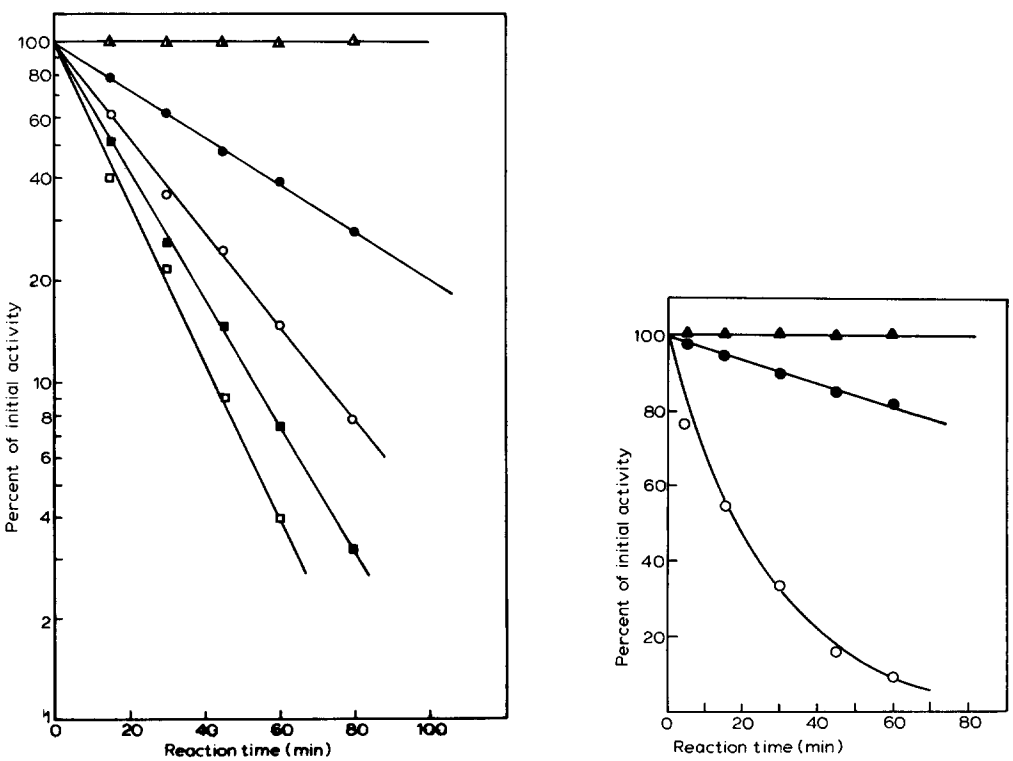

Fig. 1. Inactivation of adenosine deaminase by reagent I, reagent II and bromoacetate at pH 8. Enzyme, without alkylating reagent (▲); 1 mM bromoacetate added (△); 1 mM reagent added (●); 0.3 mM (○), 0.6 mM (■) and 1 mM reagent II added (□).

Fig. 2. Protection of purine riboside against adenosine deaminase inactivation by reagent II at pH 8. Enzyme, without reagent II or purine riboside (▲); 1 mM reagent II added (○); 1 mM reagent II and 100 μM purine riboside added (●).

TABLE I

EXPERIMENTAL DATA CONCERNING THE REACTION OF ADENOSINE DEAMINASE WITH REAGENT II AT pH 8

Values of inactivation are given before Sephadex G-25 column. Bound reagent means mol reagent per mol enzyme.

Expt. No.	Before affinity column		After affinity column		CM-Amino acid residues per mol 100% inactive enzyme	
	Inactivation (%)	Bound reagent (equivalents)	Recovered active enzyme * (%)	Recovered 100% inactive enzyme ** (%)	ε-CM-Lys	CM-His
1	15	0.14	84	16	—	—
2	30	0.28	72	28	0.27	0.62
3	50	0.52	48	52	0.28	0.59
4	80	0.78	21	79	0.30	0.60

* No bound reagent was found in all experiments.
** 1 mol reagent bound per mol 100% inactive enzyme always resulted.

determined according to a previously described method [6,10]. The same method was applied to reagent II. Also in this case two isosbestic points are present in the ultraviolet spectra of the enzyme and reagent II, at 250 and 259 nm, $\epsilon = 12.8$ and $15.7 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, respectively. The first isosbestic point was utilized for the calculation of the total molar concentration (bound reagent plus enzyme). The experimental absorptions were determined at 265 and 255 nm, where the extinction coefficients are, respectively, for the enzyme 21.2 and $13.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ and for the reagent II 15.1 and $14.6 \cdot 10^3\ M^{-1} \cdot cm^{-1}$. A good relation exists between the percentage of inactivation and the equivalents of bound reagent (see Table I). The amount of reagent bound to 100% inactive enzyme was calculated on the partially inactivated enzyme (by reagent I or II), after the 'affinity column'. In this case 1 mol reagent I or II bound per mol 100% inactive enzyme was found. The active enzyme, obtained by elution with 4 mM guanylurea (pH 7.8), does not show incorporation of the inhibitor.

Identification of alkylated amino acid residues

Acid hydrolysis produces the cleavage of the amide bond between the alkylating reagent and the involved amino acid(s). The result of the cleavage consists in the production of carboxymethyl amino acid(s). According to this hypothesis, ε-CM-lysine was shown to be the amino acid involved in the alkylation of adenosine deaminase by reagent I at pH 8 [6]. In the same conditions, previously utilized to obtain the highest recovery of carboxymethyl amino acid [10], the acid hydrolysis of adenosine deaminase inactivated by reagent II at pH 8, at some extents of inactivation, was carried out. In Table I the CM-amino acid residues involved in the inactivation of adenosine deaminase are shown. It appears that in the case of reagent II also a histidine residue (as major component) is involved in the alkylation. The amount of carboxymethylamino acid(s), after a 48-h-hydrolysis, accounted for 85—90% of the expected data.

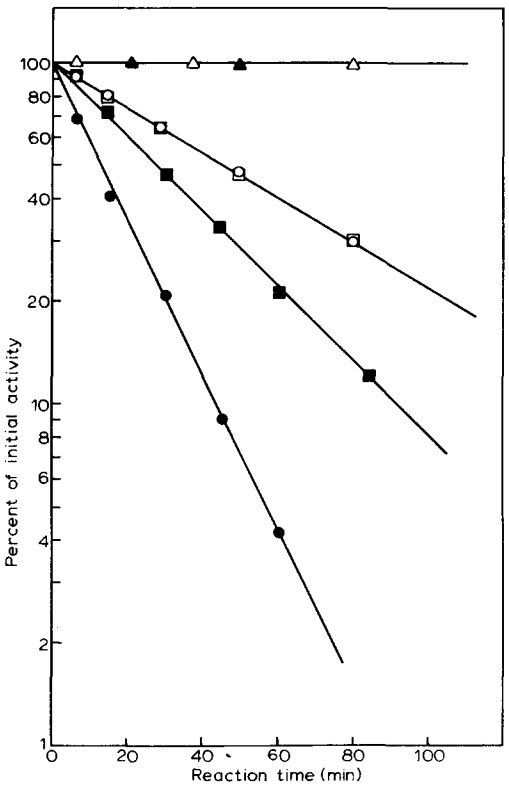

Fig. 3. Inactivation of adenosine deaminase by reagent I and II at pH 8 and 5.5. Enzyme, without alkylating reagent, at pH 8 (▲) or pH 5.5 (△); 1 mM reagent I added, pH 8 (○) or pH 5.5 (□); 1 mM reagent II added, pH 8 (●) or pH 5.5 (■).

pH effect on the alkylation of adenosine deaminase by 9-(4-bromoacetamidobenzyl)adenine (I) and 9-(2-bromoacetamidobenzyl)adenine (II)

The inactivation of adenosine deaminase by reagent I and II (1 mM) at pH 5.5 and 8 is shown in Fig. 3. No difference appears in the adenosine deaminase alkylation with reagent I, either at pH 5.5 or 8; instead, evident differences exist in the adenosine deaminase alkylation with reagent II. Different pseudo first-order rate constant ($k_2 = 4.5 \cdot 10^{-2}$ min^{-1}) resulted for reagent II at pH 5.5. Purine riboside strongly protects against inactivation of enzyme by reagent

TABLE II

DETERMINATION OF THE CM-DERIVATIVES INVOLVED IN THE ADENOSINE DEAMINASE INACTIVATION BY REAGENT I AND II AT pH 8 AND 5.5

The reported values are referred to 1 mol 100% inactive enzyme. The experimental procedure for hydrolysis and analysis is given in Methods.

Inhibitors	pH	ε-CM-lysine	CM-histidine
Reagent I	8	0.87	—
	5.5	0.79	—
Reagent II	8	0.30	0.60
	5.5	0.57	0.22

I and II also at pH 5.5. At this pH too, a good relationship was found between the percentage of inactivation and the equivalents of bound reagent I and II. 1 mol reagent I or II, bound per mol inactive enzyme at pH 5.5, was calculated in the inactive enzyme not adsorbed on the 'affinity column'.

In Table II the CM- amino acid residues involved in the inactivation of adenosine deaminase are given. In this case the 100% inactive enzyme was used. It appears that a lysine is the amino acid involved in the inactivation of adenosine deaminase with reagent I at pH 8 and 5.5. Instead, in the case of reagent II, an inversion clearly resulted in the type of residues interested in the alkylation.

Discussion

The presence of a histidine in the active site of adenosine deaminase had been formerly supposed by studies on V variation in function of pH [17] and enzyme inactivation by ethoxyformic anhydride or N-bromosuccinimide [18]. The detection of CM-histidine, as the main amino acid involved in the alkylation and inactivation of the enzyme at pH 8 by reagent II, proves the presence of a histidine in the region of the active site. This histidine was not alkylated by the more usual reagents of the enzyme alkylations, like haloacetates and related amides. This behaviour may be explained assuming that the amino acid is buried in a hardly accessible region, therefore an alkylating affinity label as reagent II is so far the only one capable of evidencing it. On the basis of these results it is not yet possible to state if the histidine alkylated by reagent II is really the same involved in catalysis (although purine riboside protects enzyme against inactivation and CM-histidine is not recovered by hydrolyzing the purine riboside protected enzyme). However, the higher alkylating efficiency of reagent II, with respect to reagent I, is probably connected with histidine alkylation.

The study of pH effect on the alkylation rate of adenosine deaminase by reagent I pointed out that a change of pH from 8 to 5.5 did not alter the alkylation rate of the lysine residue, which in both cases resulted as the only carboxymethylated amino acid present in chromatogram. All this is surprising since a change in concentration of the deprotonated form, in which the alkylants react [19], amounted to nearly 250 times, taking $pK_a = 10$ as the ϵ-aminogroup of lysine. An explanation for this peculiar reactivity of lysine could be found in the particularly low value of pK_a of the ϵ-aminogroup of lysine. This fact could be ascribed to both of the two factors: (a) lysine insertion within a hydrophobic environment, whose shape could itself cause lowering of pK_a and whose presence was previously proved [20—22]; (b) lysine insertion within a particular environment characterized by positively charged groups, whose presence was supposed on the basis of amidine derivative inhibition in discharged forms [20].

An alternative explanation for the invariability of alkylation rate with concentration of the deprotonated form could be found in a different proceeding of the alkylation reaction. The postulation concerning two stages, fast for the alkylating reagent, and slow for the ϵ-aminogroup alkylation has been reported

above [6]. However, the alkylation reaction could take place in another way, e.g. in a three-stage process, as indicated in the schema

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \overset{k_1^0}{\rightleftarrows} EI^0 \overset{k_2}{\rightarrow} EA$$

where EI° represents the likely building up of an enzyme-inhibitor complex, practically indissociable, to which the final alkylation stage could be subsequent. Instances of a similar complex were pointed out on treating adenosine deaminase with coformycin or deoxycoformycin [23]. The complex phase formation appeared slow and was characterized by its half-life period ($t_{1/2}$ = 29 h).

In the present case the slow reaction rate, which we measured, might not be attributed to the true alkylation reaction k_2, but to k_1°, concerning the second stage. This stage would be independent of pH and therefore the failed response to hydrogenionic concentration might be explained. In order to prove the presence of this complex, incubation tests were carried out with 9-(4-acetamidobenzyl)adenine, another competitive inhibitor (k_1 = 4.5 μM). Subsequent dilution and assay were pursued in operative conditions, as for the alkylating reagent I. No variation appeared in enzyme activity. Although this result induced us to discard the second hypothesis, the presence of bromine might in a way be determinant in the formation of an irreversible complex. On the contrary, a different behaviour resulted with reagent II, the other affinity label which we used. In fact, a decrement in alkylation rate was observed on varying pH between 8 and 5.5.

It is worth noting that at pH 5.5 an inversion clearly resulted in the type of residues interested in the alkylation. In fact, after hydrolysis of the pH 5 reaction product, CM-lysine and CM-histidine (0.57 and 0.22 mol, respectively) were found. This inversion proceeded parallel to a decrement in alkylation rate and proved what had been observed with reagent I, that lysine is unaffected by any pH variation. The doubling in amount of CM-lysine at pH 5.5, even though calculable by the 1.7-fold decrement in k_2, is not so clearly interpretable, since such a phenomenon arises from the addition of two reactions, one unaffected and the other affected by a pH variation.

Studies are in progress to isolate the peptide(s) containing the alkylated lysine or histidine after tryptic digestion.

Acknowledgements

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