Biochemistry (Moscow), Vol. 67, No. 7, 2002, pp. 770-777. Translated from Biokhimiya, Vol. 67, No. 7, 2002, pp. 930-938.

Interaction of Adenosine Deaminase with Inhibitors. Chemical Modification by Diethyl Pyrocarbonate

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Received June 8, 2001
Revision received March 26, 2002

Abstract—The interaction of adenosine deaminase (adenosine aminohydrolase, ADA) from bovine spleen with inhibitors—erythro-9-(2-hydroxy-3-nonyl)adenine, erythro-9-(2-hydroxy-3-nonyl)-3-deazaadenine, and 1-deazaadenosine—was investigated. Using selective chemical modification by diethyl pyrocarbonate (DEP), the possible involvement of His residues in this interaction was studied. The graphical method of Tsou indicates that of six His residues modified in the presence of DEP, only one is essential for ADA activity. Inactivation of the enzyme, though with low rate, in complex with any of the inhibitors suggests that the adenine moiety of the inhibitors (and consequently, of the substrate) does not bind with the essential His to prevent its modification. The absence of noticeable changes in the dissociation constants of any of the enzyme–inhibitor complexes for the DEP-modified and control enzyme indicates that at least the most available His residues modified in our experiments do not participate in binding the inhibitors—derivatives of adenosine or erythro-9-(2-hydroxy-3-nonyl)adenine.

Key words: adenosine deaminase, active site, histidine residues, chemical modification, adenosine deaminase inhibitors

Adenosine deaminase (adenosine aminohydrolase, ADA, EC 3.5.4.4) is one of the key enzymes in the metabolism of purine nucleosides. The enzyme catalyzes deamination of (deoxy)adenosine into (deoxy)inosine, releasing ammonia [1]; it is widespread in all tissues of vertebrates and plays an important role in the nervous [2] and vascular [3] systems and in maturation and differentiation of lymphoid cells [4]. Hereditary ADA deficiency results in severe combined immunodeficiency syndrome [5]. The enzyme level abnormalities are reported in acquired immunodeficiency syndrome [6] and in various autoimmune diseases [7, 8]. When the immune system is mobilized in infectious diseases, ADA activity increases: in clinics of some countries the ADA activity level is taken as a test in tuberculosis diagnosis [9, 10].

The mechanism of enzymatic reaction catalyzed by ADA is of special interest because some adenosine analogs are chemotherapeutic agents, and preparations of nucleoside nature are with high probability ADA substrates. Modification of such preparations in accord with the mechanism of interaction with ADA can prolong the time of their pharmacological activity. However, modulation of ADA activity by inhibitors—2′-deoxycoformycin, EHNA and its derivatives, which are substrate analogs—promotes regulation of the action of adenosine, a physiologically active substrate of the enzyme [11-13]. Thus, to understand the mechanism of the reaction catalyzed by ADA and to synthesize effective pharmaceutical preparations, it is necessary to reveal the structure of the ADA active site and amino acid residues responsible for its functioning and binding with the substrate and inhibitors.

Crystallographic study of ADA allowed determination of the main chemical aspects of enzymatic deamination [14-17]. These studies demonstrated that Zn^{2+} and also His, Gly, Glu, and Asp residues participate in enzymatic deamination of adenosine. Some of these amino acid residues have been replaced by directed mutagenesis

Abbreviations: ADA) adenosine deaminase; DEP) diethyl pyrocarbonate; DTNB) 5,5′-dithiobis(2-nitrobenzoic acid); 1-deazaAdo) 1-deazaadenosine; EHNA) erythro-9-(2-hydroxy-3-nonyl)adenine; 3-deazaEHNA) erythro-9-(2-hydroxy-3-nonyl)-3-deazaadenine.

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[17, 18]. The necessity of Glu217 and His238 for enzymatic catalysis and Cys262 for the active conformation of the enzyme was proved. The role of Trp residues in stabilization of the ADA active site is highlighted in [19, 20]. Our studies of chemical modification of Trp residues by N-bromosuccinimide in the enzyme from bovine gray and white matter proved the importance of Trp for ADA activity [21]. The data indicate that Trp residues not revealed in the X-ray study are close to the active site and are important for the catalytic event. Analogous investigations with the enzyme from bovine brain and spleen performed in the presence and in the absence of inhibitors of two different groups, adenosine and EHNA derivatives, proved the hypothesis that inhibitors of these two groups have different binding sites on the enzyme [22].

The goal of this work was to study possible participation of His residues in the interaction of ADA with inhibitors of the two mentioned groups. For this, a method of selective chemical modification by DEP was used.

MATERIALS AND METHODS

ADA from bovine spleen was isolated and purified as described earlier [23]. The electrophoretically homogeneous low-molecular-weight fraction with specific activity 350 μmole/min per mg protein was used for all experiments. ADA inhibitors—EHNA, 3-deazaEHNA, and 1-deazaAdo—were synthesized as described earlier [24-26]. Adenosine, BSA, DEP, DTNB, SDS, and NH_2OH-HCl were from Sigma (USA).

Methods for determination of the dissociation constant of the enzyme–inhibitor complex K_i and enzymatic activity were described by us earlier [22, 23].

The enzyme was modified by DEP according to Ovadi and coworkers [27] as presented in our earlier work [28]. Experiments were performed in the most ADA-favorable phosphate buffer containing 8 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4 (working buffer).

In experiments on modification of the enzyme–inhibitors complex, ADA was preincubated for 10 min in the presence of inhibitor, and then the mixture was divided into several parts and to each of them a freshly prepared DEP solution in methanol was added to a certain final concentration. The same quantity of methanol was added to the control. In certain time periods, the residual enzyme activity was evaluated as the percent of activity of the control.

The number of the SH groups in the native and DEP-treated ADA was estimated using DTNB in the presence of 0.1% SDS [29].

Reactivation of the DEP-inactivated enzyme was tested in the presence of NH_2OH-HCl. ADA (2.5 μM) was incubated in the presence of 1.8 mM DEP, then aliquots were taken at different times and placed in 17 mM imidazole buffer (pH 7.1), and the residual activity was evaluated. To a part of the treated enzyme aliquot of NH_2OH-HCl solution (pH 6.8) was added to a final concentration 0.4 M, and activity in the samples with and without NH_2OH-HCl was determined in 24 h. Since the presence of NH_2OH-HCl excludes the possibility of evaluation of enzymatic activity by the quantity of released ammonia, in these experiments ADA activity was evaluated by the difference of adenosine and inosine absorbance at 265 nm ($\Delta\varepsilon$ = 8.4 $mM^{-1}\cdot cm^{-1}$) [30]. The enzyme and substrate concentration in 2 ml of the incubation mixture in the spectrophotometer cuvette were 0.1 and 100 μM, respectively.

To study the pH dependence of the inactivation process, the enzyme was treated with DEP in 10 mM phosphate buffer at pH values from 5.7 to 9.1. The rate constant of the pseudo-first order reaction k_{obs} was determined from the time dependence of the enzyme activity at each pH value. The pK_a of inactivation reaction was determined from the pH dependence of k_{obs}.

The protein concentration was determined according to Bradford using BSA as the standard [31].

The spectral measurements were performed using a Specord M-40 spectrophotometer (Germany) with quartz thermostatted cuvettes with optical pathlength 1 cm.

The kinetic data were treated using the Enzfitter program from Biosoft (Great Britain). Statistical treatment was performed using the Excel Data Analysis program.

RESULTS

Inactivation of ADA in the presence of DEP. When ADA was incubated in DEP-containing working buffer at 23°C, inactivation of the enzyme depending both on time and reagent concentration was observed. Time dependences of ADA activities at various DEP concentrations are shown in Fig. 1a. Figure 1b displays these dependences corrected for decomposition of reagent in buffer solution using the equation [32]:

$$\ln A/A_o = -(k_{obs}/k_1)[DEP]_o(1 - \exp(-k_1 t)),$$

where A_o and A are the initial enzyme activity and activity at the moment t, respectively, k_{obs} is the pseudo-first-order rate constant of inactivation, $[DEP]_o$ is the initial DEP concentration, and k_1 is the first-order rate constant of DEP hydrolysis in the working buffer. Under our experimental conditions, k_1 determined as described in [28] was $8.1\cdot10^{-3}$ min^{-1}. Activity of the control incubated in the presence of the maximal methanol concentration used (2% v/v) is presented as curve *0*. For each DEP concentration, k_{obs} was determined graphically as the slopes of the lines in Fig. 1b.

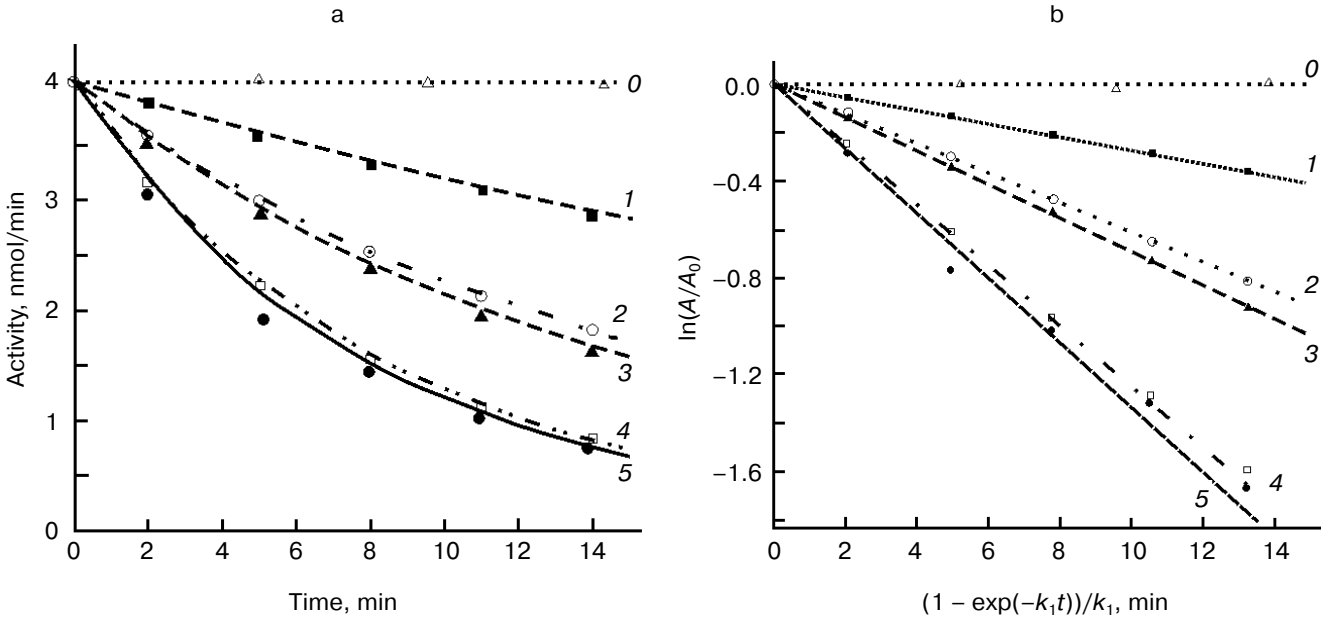

Fig. 1. a) Time dependence of ADA activity; b) the same dependences linearized as described in the text. DEP concentrations (mM): *0*) 0; *1*) 0.3; *2*) 0.63; *3*) 0.94; *4*) 1.25; *5*) 1.5. Enzyme concentration, 70 nM.

The dependence of the pseudo-first-order rate constant k_{obs} on DEP concentration is expressed by equation: $k_{obs} = k_2[DEP]^n$, where k_2 is the second-order rate constant of inactivation reaction and n is the reaction order [33]. Linearity of the plot (Fig. 2a, curve *1*) indicates that under our reaction conditions, formation of reversible enzyme–reagent complex does not occur [32]. Determined graphically as the slope of this line, k_2, the second-order rate constant of ADA inactivation was 91 $M^{-1}\cdot min^{-1}$.

Double logarithmic dependence of k_{obs} on DEP concentration is plotted in Fig. 2b (curve *1*). The order of

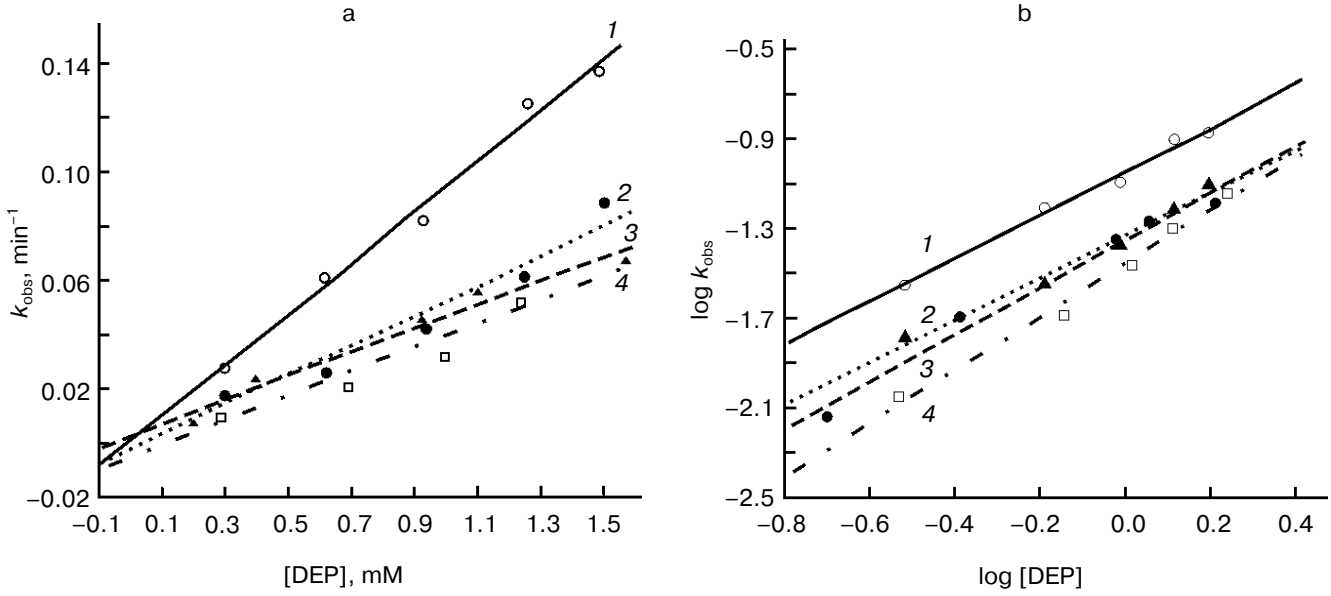

Fig. 2. a) Pseudo-first-order rate constant of ADA inactivation (k_{obs}) versus DEP concentration; b) double logarithmic dependence of k_{obs} on DEP concentration: *1*) enzyme without additions; *2*, *3*, *4*) before treatment with DEP, enzyme was incubated in the presence of 1-deazaAdo (8), EHNA (20), and 3-deazaEHNA (30), respectively (the ratios of inhibitor concentration to K_i are given in the parentheses). ADA concentration, 70 nM.

reaction *n* was estimated as the slope of this curve and appeared to be equal to 1; this indicates that the DEP–ADA interaction stoichiometry is equimolar.

Selectivity of modification of histidine residues. *The value of the second-order rate constant of ADA inactivation* in the presence of DEP, 91 $M^{-1}\cdot min^{-1}$, is within the limits typical of interaction of reagent with His [34]. The second-order rate constant of DEP interaction with other amino acid residues is significantly lower.

Increase in absorbance at 240 nm depending on incubation time was observed *in differential absorbance spectra* of ADA solution containing DEP in methanol, registered against the control (enzyme solution contains only methanol) (spectra not presented here). In experiments performed in phosphate buffer, absorbance at 240 nm is typical of ethoxyformyl derivatives of histidine [35, 36] and the absence of negative absorbance at 280 nm indicated that at least at DEP concentration used by us, O-acetylation or O-alkylation of tyrosine residues did not occur [37].

Role of the SH groups in ADA inactivation. We know from the literature and our own investigations that the SH groups are not crucial for ADA activity [38]. However, on evaluation of their number in preparations inactivated with DEP to 20% residual activity, all the SH groups determined in the native protein (5 moles per mole protein molecule) appeared to be oxidized. To be sure that oxidation of the SH groups does not contribute to ADA inactivation, the following experiment was performed. Enzyme preparation was pretitrated with DTNB to molar ratios undoubtedly exceeding those needed for blocking accessible SH groups. After each addition of DTNB, the enzyme was incubated for 40 min at 25°C. Then an aliquot was taken from the incubated mixture, its activity was estimated, and DEP was added to final concentration 2.5 mM. Activity of the enzyme modified in the presence of DTNB was again estimated after 15 min. Dependences of the enzyme activity on molar ratio [DTNB]/[ADA] before (curve *1*) and after addition of DEP (curve *2*) are presented in Fig. 3. Curve *1* demonstrates that at any DTNB/ADA ratio, activities of the enzyme with the blocked SH groups and the initial preparations are equal. At any DTNB concentration, addition of DEP decreased ADA activity to 10-11% of its initial value (curve *2*). Thus, inactivation of ADA in the presence of DEP is not a result of modification of the SH groups.

Reactivation of the DEP-modified enzyme in the presence of NH_2OH-HCl is considered as evidence for the responsibility of His for the observed inactivation [39]. In our experiments, analysis of ADA samples incubated with NH_2OH-HCl for 24 h after inactivation by the reagent (see "Materials and Methods") showed reactivation of the enzyme to 70-100% of control activity. In the samples not treated with NH_2OH-HCl, activity remained on the level noted after treatment with DEP, that is, spontaneous reactivation did not occur.

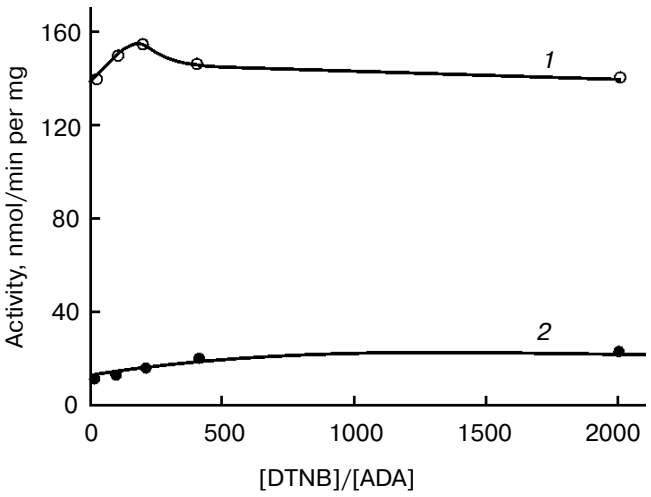

Fig. 3. ADA activity (0.7 nM) at various [DTNB]/[ADA] molar ratios before (*1*) and after (*2*) addition of DEP to final concentration 2.5 mM.

Dependence of inactivation on pH. It is known that DEP interacts with non-protonated imidazole groups and selective reaction of DEP with His occurs between pH 5.5 and 8.0 [34]. The pH dependence of k_{obs} obtained at DEP concentration 0.8 mM is presented in Fig. 4 (curve *1*). A theoretical curve obtained for pK_a 7.8 coincides well with experimental points on the plot; this value is near the

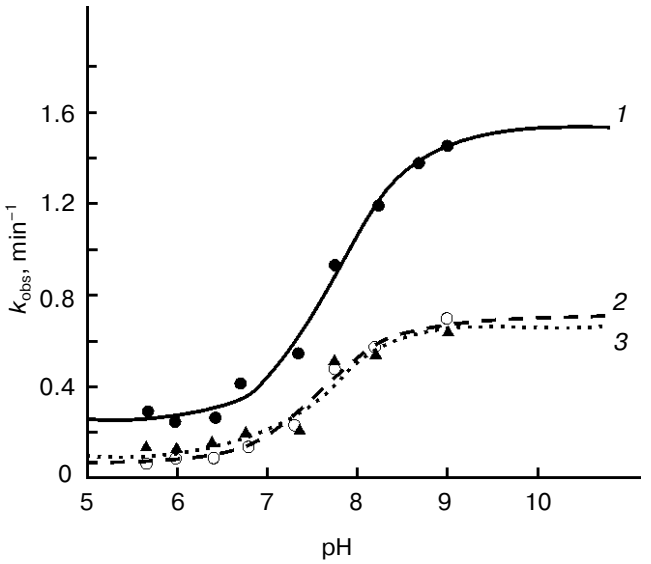

Fig. 4. pH dependence of k_{obs} of ADA inactivation reaction in the presence of DEP: *1*) enzyme without inhibitor; *2*, *3*) enzyme in the presence of 83 μM 1-deazaAdo and 2 μM EHNA, respectively. Concentrations: 0.8 mM DEP, 0.1 μM enzyme. Points correspond to experimental data, theoretical curves were obtained for pK_a 7.8 (*1*), 7.7 (*2*), and 7.6 (*3*).

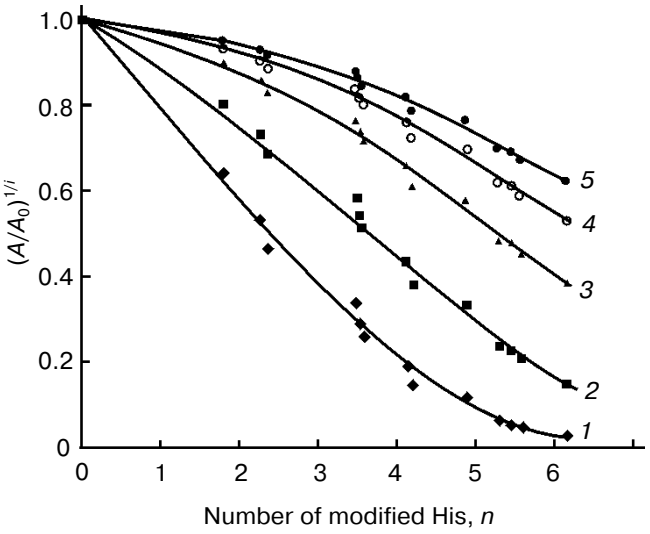

Fig. 5. Determination of the number of modified His essential for ADA activity. *i* values: *1*) 0.5; *2*) 1; *3*) 2; *4*) 3; *5*) 4.

upper limit of pK_a values for histidine in proteins known from literature. It should be noted that such increase in pK_a of His can be caused by their being in a neighborhood with acidic amino acid residues [34, 35, 40].

Thus, all experiments described above indicate that loss in ADA activity observed in the presence of DEP is a result of modification of His.

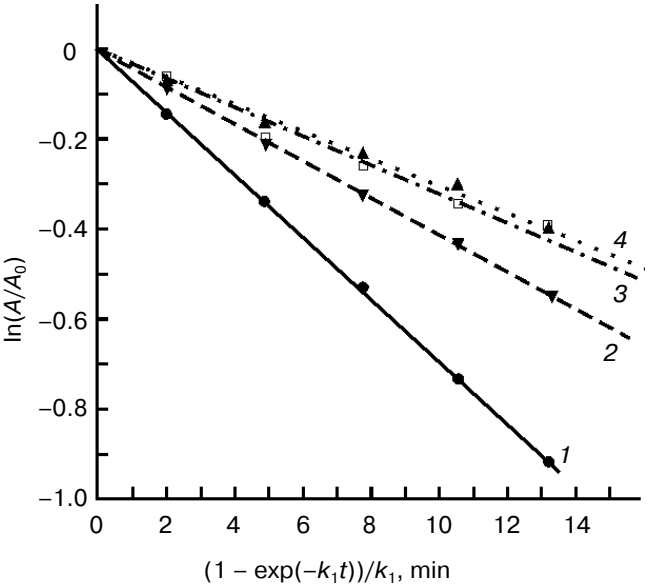

Fig. 6. Inactivation of ADA in the presence of inhibitors. Corrected time dependences of residual activity on incubation in the presence of 1 mM DEP: *1*) free ADA; *2*, *3*, *4*) ADA complex with 1-deazaAdo, EHNA, and 3-deazaEHNA, respectively. ADA concentration and inhibitor concentration/K_i ratios are the same as those given in Fig. 2a legend.

Number of His residues essential for ADA activity. To evaluate the number of essential His residues, the graphical method of Tsou was used [41]. Dependences of A/A_o raised to the power $1/i$ (i is a varied number of the modified amino acid residues essential for the enzyme activity) on the number of modified His at i values of 0.5, 1, 2, 3, and 4 are displayed in Fig. 5. The number of modified residues was determined by absorbance intensity at 240 nm in differential spectra described above. The presented data are limited by DEP concentration 1.5 mM; at this concentration, six His are modified. Higher reagent concentrations resulted in appearance of turbidity and/or short-wavelength shift of the UV maxima; this indicated some more severe and uncontrolled changes in the protein molecule.

Of the presented curves, curve *2* corresponding to $i = 1$ is the closest to linearity. Thus, of six His modified in the presence of DEP, only one His per enzyme molecule is essential for the activity of ADA from bovine spleen.

Reaction of ADA with DEP in the presence of inhibitors. Figure 6 shows the time dependences of activity of free ADA (curve *1*) and ADA complexes with inhibitors (1-deazaAdo, EHNA, and 3-deazaEHNA, curves *2*, *3*, and *4*, respectively) incubated in the presence of 1 mM DEP. Decrease in slopes of curves *2-4* (decrease in k_{obs}) indicates reduction of inactivating action of DEP by inhibitors, the protective effect of EHNA and 3-deazaEHNA being manifested more clearly compared with 1-deazaAdo. This corresponds with efficiency of ADA inhibition by the mentioned compounds. The dissociation constants of the complexes of these compounds with ADA from bovine spleen are given in the second column of Table 1.

We investigated the inactivation process of ADA complexes with the different inhibitors in the presence of DEP (see "Materials and Methods"). Analogously to experiments with free protein described above (Fig. 1), the enzyme activities versus time were plotted at various DEP concentrations. For the studied complexes, k_{obs} were determined from the plots. The values of k_2, the second-order rate constant of ADA inactivation reaction were estimated by slopes of the curves of k_{obs} versus DEP concentration (Fig. 2a, curves *2-4*). Parameters of inactivation reaction in the presence of DEP of free ADA and ADA complexes with inhibitors are compared in Table 1. As shown, the second-order rate constants determined from these slopes decreased to 53 and 43 $M^{-1}\cdot min^{-1}$ for enzyme complexes with 1-deazaAdo, EHNA, and 3-deazaEHNA, respectively. Decrease in k_2 proves decrease in inactivating effect of modifier by inhibitors. It should be noted that k_2 meanwhile remains within the limits typical of interaction between DEP and His [35].

The degree of change in this parameter proves a conclusion based on Fig. 6 that inhibitors of EHNA group protect the enzyme from inactivation in the presence of DEP more efficiently than 1-deazaAdo.

Table 1. Effect of inhibitors on characteristics of adenosine deaminase inactivation by diethyl pyrocarbonate

Inhibitor	K_i, μM	[I]/K_i	k_2, $M^{-1}\cdot min^{-1}$	n	pK_a
Without inhibitor	—	0	91	1.0	7.8
1-Deazaadenosine	10	8	53	0.97	7.7
EHNA	0.09	22	42.7	1.08	7.6
3-DeazaEHNA	0.13	30	42.6	1.2	not defined

Note: [I], inhibitor concentration, μM; k_2, the second-order rate constant; n, the order of reaction.

The order of ADA inactivation reaction (n) evaluated from double logarithmic dependences of k_{obs} on DEP concentration (Fig. 2b, curves *2-4*) in each of the studied complexes is close to unity (Table 1).

The pH dependence of inactivation of the enzyme–inhibitor complex was studied under conditions analogous to those described for free enzyme. The pH dependences of k_{obs} for enzyme complexes with 1-deazaAdo (curve *2*) and with EHNA (curve *3*) are presented in Fig. 4 along with pH dependence for free enzyme (curve *1*). Analogously to the order of reaction, the pK_a value for inactivation of the enzyme–inhibitor complexes remains essentially the same as that for the native enzyme (Table 1).

Analysis of data reported shows that in the enzyme–inhibitor complexes as well as in free enzyme, inactivation is a consequence of His modifications.

Interaction of modified ADA with inhibitors. The experimental results described above indicate that the inhibitors decrease the inactivating effect of DEP on ADA. However, parameters of the process indicate that His modification is the reason for inactivation of ADA both in complexes with the studied compounds and in the free state. These contradictory data prevent an unambiguous conclusion about possible screening of His residues in the active site of ADA by inhibitors and consequently, about participation of this amino acid in formation of the ADA–inhibitor complex.

To determine whether His residues participate in ADA interaction with inhibitors, the inhibition constants of the partly modified enzyme were determined for 1-deazaAdo, EHNA, and 3-deazaEHNA. The enzyme was preincubated in the presence of DEP. The reagent concentration and incubation time were adjusted so that the residual activity was approximately half of the control activity. A part of the studied preparation incubated in the presence of that amount of methanol (usually not more than 2% v/v), in which DEP added to the working sample is dissolved, was used as a control in each experiment.

Aliquots of working and control samples were 100-times diluted in the buffer containing inhibitors, preincubated for 10 min, and their activities were determined. To determine the dissociation constants of the enzyme–inhibitor complexes, the dependence of the enzyme activity on inhibitor concentration at two substrate concentrations was studied [42]. Values of K_i presented in Table 2 are the averages obtained in six independent experiments.

The Student's coefficients given in the last column of Table 2 indicate that there is no reliable difference between the dissociation constants of complexes of the control enzyme and that inactivated to 45% of the initial activity with all three investigated inhibitors.

Table 2. Comparison of dissociation constants of the studied compounds for adenosine deaminase, control (C) and modified (M) by diethyl pyrocarbonate

Inhibitor	A/A_0, %	K_i, μM		Student's coefficient, p
		C	M	
1-Deazaadenosine	46.3 ± 5.6	7.0 ± 2.2	7.4 ± 2.1	0.68
EHNA	42.0 ± 6.5	0.08 ± 0.01	0.07 ± 0.009	0.13
3-DeazaEHNA	46.4 ± 6.4	0.14 ± 0.03	0.11 ± 0.02	0.2

Note: ±, standard uncertainty value.

DISCUSSION

The role of histidine residues in ADA activity is widely investigated. Despite important results obtained by X-ray structural analysis [14-17] and directed mutagenesis [17, 18] mentioned above, for many laboratories chemical modification by DEP still remains the most available method providing information about the role of His in ADA activity. Earlier modification by DEP allowed us to show its participation in functioning of ADA from five regions of bovine brain [38]; these results were later proved [43]. The results of kinetic and thermodynamic investigations of the effect of DEP on the active site of ADA from small intestine using UV-spectrophotometry and isothermal titration calorimetry have been published recently [44].

It is known that in the complexes with adenosine derivatives the inhibitory activity of a compound is caused by changes in groups of the purine ring participating in the enzymatic catalysis [45]. The inhibition by EHNA and its derivatives is most likely determined by conformational changes (caused by their hydrophobic chain) in a protein molecule hindering positioning of the purine part of the molecule in the active site of enzyme.

Based on significant difference between these mechanisms, it should be expected that modification of amino acid positioned in the active site will change ADA interaction with inhibitors of these two groups in a different way.

In our experiments, EHNA and 3-deazaEHNA really possessed a larger screening effect than 1-deazaAdo. This effect results in decreased inactivation of the enzyme forming a complex with inhibitors (Figs. 6 and 2a); this seems to be due to the screening effect of conformational changes proceeding in the ADA molecule on its interaction with the inhibitors.

Our results demonstrated that of six His modified in the presence of DEP, only one is essential for ADA activity. This is in contradiction with [44], where the results of thermodynamic titration of ADA from small intestine in a wider range of DEP concentrations were interpreted as modification of three His important for activity. The authors consider that those three His which coordinate Zn^{2+} are modified.

We are inclined to believe that His238 is the histidine residue essential for the enzymatic catalysis of ADA that is modified in our experiments. This is proved by the following considerations. First, the pK_a of the His responsible for ADA inactivation in the presence of DEP is high (Fig. 4). This indicates that this residue is close to acidic amino acids [40]. It should be noted that it is known from the X-ray data that His238 is in the immediate neighborhood of two acidic amino acid residues, Asp295 and Asp296 [16, 17]. Second, in our experiments ADA inhibitors having sufficiently high affinity to the enzyme (EHNA, 3-deazaEHNA, and 1-deazaAdo) did not protect histidine from the action of DEP. Along with this, the inhibition constants of all these compounds did not change significantly as a result of modification of His. Actually, the His essential for activity is not protected from the action of DEP in the complex with inhibitors. However, Bhaumik et al. demonstrated that change in His238 participating in catalysis of deamination for Glu and Arg does not noticeably influence the enzyme affinity to the substrate (adenosine) and inhibitor (deoxycoformycin) [18]. This supports our suggestion that it is His238 which is attacked by the reagent in the active site.

So, our results prove the suggestion that the adenine moiety of inhibitors (and the substrate) do not bind to the histidine residue of the active site (His238). They also indicate that none of the six modified His (there are nine His in the protein molecule [46]) participates in binding inhibitors—derivatives of adenosine or EHNA.

This study was performed in the framework of the theme 2000-716 established by the Ministry of Science of the Republic of Armenia with the help of a grant for reagents of the International Union of Neurochemistry given to A. A. Antonyan. We also are grateful to G. A. Avakyan for technical assistance in the use of computer programs.

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