Mechanisms of Inactivation of Human *S*-Adenosylhomocysteine Hydrolase by 5′,5′,6′,6′-Tetradehydro-6′-deoxy-6′-halohomoadenosines[†]

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ABSTRACT: In an effort to design more specific and potent inhibitors of *S*-adenosylhomocysteine (AdoHcy) hydrolase, we investigated the mechanisms by which 5′,5′,6′,6′-tetradehydro-6′-deoxy-6′-halohomoadenosines (X = Cl, Br, I) inactivated this enzyme. The 6′-chloro (**a**) and 6′-bromo **(b)** acetylenic nucleoside analogues produced partial (∼50%) loss of enzyme activity with a concomitant (∼50%) reduction of E-NAD$^+$ to E-NADH. In addition, Ade and halide ions were released from the inhibitors in amounts suggestive of a process involving enzyme catalysis. AdoHcy hydrolase, which was inactivated with compound **a**, was shown to contain 2 mol of the inhibitor covalently bound to Lys318 of two subunits of the homotetramer. These data suggest that the enzyme-mediated water addition at the 5′ position of compound **a** or **b** produces an α-halomethyl ketone intermediate, which is then attacked by a proximal nucleophile (i.e., Lys318) to form the enzyme−inhibitor covalent adduct (lethal event); in a parallel pathway (nonlethal event), addition of water at the 6′ position produces an acyl halide, which is released into solution and chemically degrades into Ade, halide ion, and sugar-derived products. In contrast, compound **c** completely inactivated AdoHcy hydrolase by converting 2 equiv of E-NAD$^+$ to E-NADH and causing the release of 2 equiv of E-NAD$^+$ into solution. Four moles of the inhibitor was shown to be tightly bound to the tetrameric enzyme. These data suggest that compound **c** inactivates AdoHcy hydrolase by a mechanism similar to the acetylenic analogue of Ado described previously by Parry et al. [(1991) *Biochemistry 30*, 9988−9997].

AdoHcy[1] hydrolase (EC 3.3.1.1) catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and homocysteine (Hcy) (*1*, *2*). This enzyme regulates all *S*-adenosylmethionine (AdoMet)-dependent transmethylations by controlling the intracellular levels of AdoHcy, which is a potent inhibitor of AdoMet-dependent methyltransferases (*1*, *2*). Thus, AdoHcy hydrolase has become an attractive target for the design of antiviral, antiparasitic, antiarthritic, immunosuppressive, and antitumor agents (*1*, *2*).

Recently, X-ray crystal structures of the human (*3*) and rat (*4*) AdoHcy hydrolases have been solved. These structures provide for the first time insights into the nature of the amino acid residues used by AdoHcy hydrolase to catalyze the various chemical transformations proposed by Palmer and Abeles (*5*, *6*). The first reaction catalyzed by AdoHcy hydrolase involves oxidation of the 3′-OH group of the substrate AdoHcy (or Ado) by E-NAD$^+$ to form E-NADH and 3′-keto-AdoHcy (or 3′-keto-Ado) (3′-oxidative step). The C4′-proton is then abstracted, presumably by a water molecule at the active site of the enzyme, which has been activated by hydrogen-bonding interactions with Asp131 and His55 (*3*) (4′-proton abstraction step), followed by *β*-elimination of Hcy (or water) to form 3′-keto-4′,5′-didehydro-5′-deoxy-Ado. His301 is then proposed (*3*) to participate in the activation of a water molecule (or Hcy) at the active site, which adds in a Michael-type addition to the 4′,5′ double bond (5′-hydrolytic step), generating 3′-keto-Ado (or 3′-keto-AdoHcy). Reduction of 3′-keto-Ado (or 3′-keto-AdoHcy) by E-NADH yields Ado (or AdoHcy), which is then released from the active site of the enzyme (3′-reductive step); thus, completing the catalytic cycle. The 5′-hydrolytic activity (also referred to as 5′/6′ hydrolytic activity) has been shown to function independently of the 3′-oxidative activity (*7*−*9*).

In the past, significant effort has been made to design potent and selective inhibitors of AdoHcy hydrolase (*1*, *2*). Most inhibitors are Ado analogues that are irreversibly oxidized to their 3′-keto derivatives with concomitant conversion of the NAD$^+$-form of the enzyme (active) to the NADH-form (inactive). These inhibitors are referred to as type I mechanism-based inhibitors. However, significant effort has also been made to identify type II mechanism-based inhibitors that are designed to be transformed by the

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[1] Abbreviations: Ade, adenine; Ado, adenosine; AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; BDDFHA, 6′-bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine; DHCeA, 2′,3′-dihydroxycyclopent-4-enyladenine; E-NAD$^+$, enzyme bound NAD$^+$; E-NADH, enzyme bound NADH; FAB, fast-atom bombardment; Hcy, homocysteine; HPLC, high-performance liquid chromatography; IC, ion chromatography; MW, molecular weight.

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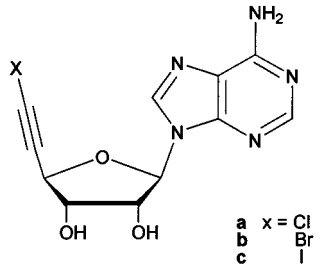


FIGURE 1: Structures of the 6′-halo-5′,5′,6′,6′-tetradehydro-6′-deoxyhomoadenosines.

enzyme to chemically reactive intermediates that would then covalently modify the enzyme. For example, Parry et al. (*10*) showed that an acetylenic analogue of homoAdo, after oxidation by the enzyme, forms a covalent linkage with AdoHcy hydrolase. This enzyme−inhibitor adduct is very acid-labile (*10*). We (*11*, *12*) recently reported that 6′-bromo-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine (BDDF-HA) can function as a substrate for the enzyme's 5′/6′-hydrolytic activity, yielding homoAdo 6′-carboxyl fluoride, which reacts with Arg196 to form a stable enzyme−inhibitor adduct.

Recently, three haloacetylenic nucleoside analogues derived from Ado (Figure 1) were synthesized in our laboratories and shown to be inhibitors of AdoHcy hydrolase (*13*). We now have elucidated the mechanisms by which these haloacetylenic nucleoside analogues inactivate AdoHcy hydrolase. The data presented in this paper clearly show that the 6′-chloro (**a**) and 6′-bromo (**b**) acetylenic nucleoside analogues are substrates for the 5′/6′-hydrolytic activity of the enzyme, generating reactive intermediates that irreversibly inactivate the enzyme through covalent modification (type II inhibition). In contrast, the 6′-iodo (**c**) acetylenic nucleoside analogue inactivates the enzyme by a mechanism similar to that observed by Parry et al. (*10*) for the acetylenic analogue of homoAdo. This involves the 3′-oxidative activity of the enzyme, and leads to the formation of covalent adducts that are labile to hydrolysis upon denaturation at neutral pH.

MATERIALS AND METHODS

Materials. Ado, Hcy, AdoHcy, NAD^+, and NADH were obtained from Sigma (St. Louis, MO). Standard samples of chloride ion, bromide ion, and iodide ion were purchased from P. J. Cobert (St. Louis, MO). Recombinant human placental AdoHcy hydrolase was overexpressed, purified, and reconstituted into the NAD^+ form as described previously (*7*). 5′,5′,6′,6′-Tetradehydro-6′-deoxy-6′-halo (Cl, Br, I) homoadenosines were synthesized as described by Robins et al. (*13*). Bromoacetaldehyde was prepared by distillation of bromoacetaldehyde diethyl acetal (Fisher Scientific, Pittsburgh, PA) from 5% sulfuric acid (*14*).

Assay of AdoHcy Hydrolase Activity. The activity of AdoHcy hydrolase was assayed in the synthetic direction as previously described (*7*) by measuring the production of AdoHcy from Ado (1 mM) and Hcy (5 mM) in 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA (buffer A). AdoHcy was analyzed by HPLC using a C18 reversed-phase column (Vydac Inc., Hesperia, CA) with absorbance monitored at 260 nm.

Inactivation of AdoHcy Hydrolase by 6′-Haloacetylenic Nucleoside Analogues. The time-dependence of the inactivation of AdoHcy hydrolase was determined by incubating 25 nM of the enzyme with 100 μM of the inhibitor in buffer A at 37 °C for different times (e.g., 0, 5, 10 15, or 20 min). The residual enzyme activity was measured as described above. Pseudo-first-order rate constants (k_{app}) for enzyme inactivation were calculated by fitting the curves of residual enzyme activity versus time to a first-order exponential decay. The apparent binding affinities of the inhibitors (K_i) and the inhibition rate constants (k_2) were calculated using the k_{app} values and inhibitor concentrations by the method of Kitz and Wilson (*15*) according to the following equation:

$$\frac{1}{k_{app}} = \frac{1}{k_2} + \frac{K_i}{k_2}\cdot\frac{1}{[I]}$$

Determination of Halide Ion Released from the 6′-Haloacetylenic Nucleoside Analogues. The amounts of halide ion released from the 6′-haloacetylenic nucleoside analogues upon incubation with AdoHcy hydrolase were determined by ion chromatography (IC). The enzyme was incubated with inhibitor under the experimental conditions described above. An aliquot (containing 200 μg of protein) of the reaction mixture was then withdrawn; the protein was denatured by adding absolute ethanol (3×, v/v) and the protein precipitate was removed by centrifuging at 10000*g* for 5 min. The pellet was washed twice with 500 μL of double-distilled water. The washes were pooled with the supernatant and passed through a Bakerbond Sep C18 micro column (J. T. Baker, Philipsburg, NJ). The filtrate and washes (2 mL) were combined and lyophilized and residue was dissolved in 100 μL of water. For analysis of chloride and bromide ions, a sample (50 μL) was injected onto a IonPac AS14 column (Dionex, Sunnyvale, CA) attached to a Dionex IC 500 system. The anion was eluted isocratically (3.5 mM sodium carbonate, 1 mM sodium bicarbonate) at a flow rate of 1.2 mL/min. Iodide ion was analyzed using the method of Bradfield and Cooke (*16*) [C18 reversed-phase column (ODS Hypersil 250 × 4.6 mm. Alltech, Deerfield, IL), 0.5 mM tetrabutylammonium hydroxide/5% CH_3OH (pH 7.1 adjusted with 0.1 M potassium hydrogen phthalate), flow rate of 1.5 mL/min, detection at 260 nm]. Quantitation of the halide ions was performed with standard curves constructed from known halide ion concentrations. The assay recoveries were 95−100% in the control experiments using bovine albumin and halide ion standards.

Determination of Formation of Adenine from the 6′-Haloacetylenic Nucleoside Analogues. AdoHcy hydrolase was incubated with an inhibitor under the experimental conditions described above. An aliquot (containing 100 μg of protein) of the reaction mixture was then withdrawn and the reaction was terminated by addition of 10 μL of 5 M $HClO_4$. The precipitate was removed by centrifugation and the supernatant was analyzed for Ade by HPLC with a reversed-phase C18 column as described previously (*8*). The HPLC peak assigned to Ade was verified by co-injection of a standard sample of Ade. Further confirmation of this structural assignment was obtained by mass spectrometry (data not shown).

Analysis of the Cofactor Bound to AdoHcy Hydrolase. The extent of conversion of NAD^+ to NADH upon incubation with an inhibitor was analyzed using fluorescence spectroscopy as described previously (*8*). Addition of 3 vol of 97%

ethanol to the protein sample (0.1−0.2 mg) was used to release the tightly bound cofactors (NAD^+, NADH) from the enzyme. After centrifugation at 10000*g* for 5 min at 4 °C, the pellet was washed with 97% ethanol and centrifuged again. Pooled supernatants were lyophilized in the dark. The residue was then dissolved in 1.0 mL of 60 mM sodium pyrophosphate buffer (pH 8.8) containing 0.3% semicarbazide. The NADH content was determined by fluorescence spectrometry (460 nm emission, 340 nm excitation) using a standard curve generated from known amounts of NADH. The NAD^+ content was determined after its conversion to NADH by addition of 10 μL of a 1% solution of bakers' yeast alcohol dehydrogenase (Sigma, St. Louis, MO) and 20 μL of 97% ethanol.

Analysis of the Cofactor Released from AdoHcy Hydrolase. AdoHcy hydrolase (10 μM) was incubated at 37 °C for 20 min with 100 μM of 2′,3′-dihydroxycyclopent-4-enyladenine (DHCeA), or compound **a** or **c**. An aliquot (300 μL) of the reaction mixture was transferred to a Millipore (Bedford, MA) Biomax-5 centrifugal filter (MW 5000 cutoff) and centrifuged at 5000*g* for 10 min. The filtrate (200 μL) was then assayed for NAD^+/NADH content using the fluorescence method described above.

Stoichiometries of Total Binding and Covalent Binding of the 6′-Haloacetylenic Nucleoside Analogues to AdoHcy Hydrolase. AdoHcy hydrolase (0.5 mg) in 250 μL of buffer A was incubated with 100 μM of compound **a** or **c** at 37 °C for 5 min. The reaction mixture was then applied to a Millipore Biomax-5 centrifugal filter (MW 5000 cutoff). The concentration of free inhibitor in the filtrate was assayed by HPLC. The concentration of enzyme-bound inhibitor was obtained by subtracting the concentration of free inhibitor from the total concentration added to the incubation mixture. The ratio of moles of bound inhibitor per mole of enzyme subunit is referred to as the total binding.

To determine the extent of covalent binding, AdoHcy hydrolase (1.0 mg) in 1.0 mL of buffer A was incubated with 500 μM of the inhibitor at 37 °C for 2 h. The reaction mixture was then passed through a Sephadex G-25 spin column to remove unbound inhibitor. The enzyme−inhibitor complex was denatured with 8 M urea and passed again through a Sephadex G-25 spin column to remove any noncovalent-bound inhibitor as well as to change the buffer to 50 mM potassium phosphate (pH 8.0). The protein concentration was determined by the Bradford method (*17*). The inhibitor−enzyme adduct was then digested with trypsin. An aliquot of tryptic peptides corresponding to 50 μg of enzyme was transferred to a glass test tube and lyophilized. The sample was redissolved in 100 μL of water and derivatized by the method of Secrist et al. (*14*). Briefly, 100 μL of 1 M sodium acetate (pH 4.5) and 20 μL of 1 M bromoacetaldehyde were added to the sample and the tube was sealed and heated at 90 °C for 15 min. Quantitation of the amount of 1,N^6-etheno derivative of adenine formed was accomplished by fluorescence spectrophotometry (excitation at 300 nm and emission at 405 nm). A standard curve was constructed using known amounts of Ado and the derivatization procedure described above.

Isolation and Identification of Inhibitor-Labeled Peptides. Inhibitor-labeled protein was prepared by incubating the enzyme (1.0 mg in 1.0 mL buffer A) with 500 μM of the inhibitor as described above. The protein was digested with trypsin, and the peptide fragments were separated using a C18 reversed-phase HPLC column as described previously (*11*). Three detector channels were used to monitor the eluted peptides: absorbance at 215 nm, absorbance at 260 nm, and fluorescence emission at 340 nm with excitation at 280 nm. Peptide peaks exhibiting absorbance at 260 nm were collected. Aliquots were withdrawn from each sample and their inhibitor content was analyzed after derivatization with bromoacetaldehyde as described above. Mass spectra for inhibitor-labeled peptide were collected by FAB mass spectrometry and analyzed using the method of Yao et al. (*18*) employing a GPMAW program (Lighthouse Data, Odense, Denmark).

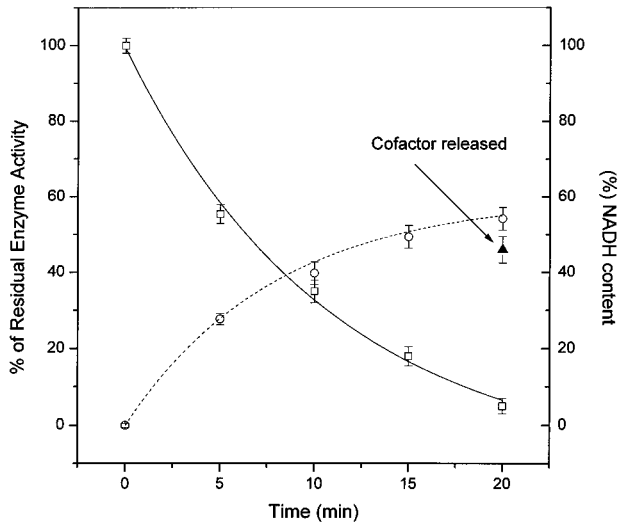


FIGURE 2: Time course of residual enzyme activity, formation of E-NADH, and release of cofactor upon incubation of AdoHcy hydrolase with compound **c**. AdoHcy hydrolase (1.0 mg/mL, NAD^+ form) was incubated with 100 μM compound **c** in buffer A at 37 °C for the indicated times. Samples containing 2 μg (residual enzyme activity), 200 μg (NADH content), and 200 μg (release of cofactor) of protein were withdrawn and used for indicated assays as described in the Materials and Methods. (□) Solid line, % of residual enzyme activity; (○) dashed line, % of NADH content; (▲) % ratio of released cofactor to enzyme.

RESULTS

General Characteristics of the Inactivation of AdoHcy Hydrolase by the 6′-Haloacetylenic Nucleoside Analogues. Recombinant human AdoHcy hydrolase was inactivated upon incubation with the 6′-haloacetylenic nucleoside analogues in both concentration-dependent and time-dependent processes (*13*). Compounds **a** and **b** produced approximately 50% loss of enzyme activity, whereas compound **c** produced complete inactivation of AdoHcy hydrolase. Using the method of Kitz and Wilson (*15*), the following kinetic parameters describing these inactivation processes were determined: compound **a**, $K_i = 1.7 \pm 0.2$ μM, $k_2 = 0.48 \pm 0.05$ min^{-1}; compound **b**, $K_i = 3.8 \pm 0.3$ μM, $k_2 = 0.49 \pm 0.3$ min^{-1}; compound **c**, $K_i = 1.10 \pm 0.04$ μM, $k_2 = 0.095 \pm 0.004$ min^{-1} (*13*). Incubation of AdoHcy hydrolase with compounds **a** and **b** also resulted in the formation of Ade and halide ions (chloride and bromide) (*13*). In contrast, no release of iodide ion or Ade was detected for compound **c** (*13*).

*Effects of Compound **c** on the Formation of NADH and Release of the Cofactor*. As shown in Figure 2, incubation

Table 1: Stoichiometry of Total Binding and Covalent Binding of Compounds **a** and **c** to AdoHcy Hydrolase

compd	total binding[a] (mol of inhibitor/ mol of enzyme subunit)	covalent binding[b] (mol of inhibitor/ mol of enzyme subunit)
c (X = I)	0.90 ± 0.02	0.070 ± 0.005 0.91 ± 0.04[c]
a (X = Cl)	0.93 ± 0.04	0.50 ± 0.02

[a] AdoHcy hydrolase (0.5 mg) in buffer A (250 μL) was incubated with 100 μM inhibitor at 37 °C for 5 min. The concentration of free inhibitor in solution was determined and the stoichiometry of the total binding was calculated as described in the Materials and Methods. [b] AdoHcy hydrolase (1.0 mg) in buffer A (1.0 mL) was incubated with 500 μM inhibitor at 37 °C for 2 h. The enzyme−inhibitor complex was isolated and the stoichiometry of covalent binding was determined after denaturation of the enzyme−inhibitor complex with 8 M urea before gel filtration on a spin column as described in the Materials and Methods. [c] The enzyme−inhibitor complex was denatured with 8 M urea in the presence of 0.1 M LiOH.

of AdoHcy hydrolase with compound **c** resulted in the formation of E-NADH as well as the release of the cofactor into the incubation media. The enzyme fully inactivated by compound **c** retained approximately 50% of the cofactor and approximately 50% was released into the incubation media.

*Stoichiometries of Binding and Covalent Modification of AdoHcy Hydrolase by Compound **c***. Upon complete inactivation of the enzyme by compound **c**, 0.90 mol of the inhibitor were bound to one mole of enzyme subunit (Table 1). After denaturation of this enzyme−inhibitor complex with 8 M urea in 0.1 M LiOH, the stoichiometry of binding of the inhibitor (0.92 mol/subunit) did not change. In contrast, if the enzyme−inhibitor complex was denatured under neutral conditions, the stoichiometry of binding changed significantly (0.07 mol/subunit). These results suggest that each subunit is labeled with a molecule of the inhibitor, but the linkage between the enzyme and the inhibitor must be labile under neutral pH conditions. Because trypsin digestion is normally carried out under very mildly basic conditions, attempts to isolate the inhibitor-labeled peptide failed (data not shown).

*Comparison of the Relative Rates of Loss of Enzyme Activity, Depletion of NAD$^+$, and Release of Ade and Chloride Ion upon Incubation of AdoHcy Hydrolase with Compound **a***. Incubation of AdoHcy hydrolase with compound **a** resulted in enzyme inactivation, depletion of NAD$^+$, release of chloride ion and Ade. To compare the relative rates of these processes, AdoHcy hydrolase (NAD$^+$ form) was incubated with compound **a** at 25 °C. At various times, aliquots of the reaction mixtures were withdrawn and assayed for residual enzyme activity, NAD$^+$ content, and release of chloride ion and Ade using the methods described in the Materials and Methods. The results are presented in Figure 3 and Table 2. Kinetic analyses revealed that loss of enzyme activity, depletion of NAD$^+$, and release of Ade are pseudo-first-order processes having rate constants of 0.85, 0.87, and 0.015 min^{-1}, respectively. However, the release of chloride ion follows second-order kinetics; $k_{app1} = 0.85$ min^{-1} and $k_{app2} = 0.015$ min^{-1}. It is interesting to note that the rates of loss of enzyme activity, NAD$^+$ depletion, and k_{app1} for chloride ion release are all approximately equal, while the rate of k_{app2} for chloride ion release is identical to that observed for the rate of Ade release. Overall, these results suggested that compound **a** reacts by two different pathways with AdoHcy hydrolase. One pathway leads to inactivation of the enzyme by conversion of E-NAD$^+$ to E-NADH, whereas the other pathway results in degradation of the inhibitor, resulting in simultaneous release of Ade and chloride ions. The rapid process of release of chloride ion accounts for about 2 mol of chloride ion per mole of tetrameric enzyme, suggesting half-of-site covalent modification of the enzyme. Similar results were observed with compound **b** (data not shown).

*Stoichiometries of Binding and Covalent Modification of AdoHcy Hydrolase by Compound **a***. Compound **a** produces partial inactivation of AdoHcy hydrolase. Incubation of the enzyme with compound **a** results in the total binding of 0.93 mol of inhibitor/mol of enzyme subunit (Table 1). HPLC analysis of the supernatant obtained after denaturation of the inactivated enzyme showed the presence of compound **a** and Ade. The amount of Ade formed was dependent on the incubation time (data not shown). After denaturation of this enzyme−inhibitor complex with 8 M urea, the stoichiometry of binding of the inhibitor changed to 0.50 mol/subunit. These results suggest that each homotetramer binds 2 mol of the inhibitor covalently and 2 mol of the inhibitor in a noncovalent manner.

Isolation and Characterization of Inhibitor-labeled Peptide Fragments. After subjecting the inhibitor (compound **a**)-labeled protein to trypsin digestion, the tryptic peptides were separated by reversed-phase HPLC. On the basis of the results of derivatization with bromoacetalaldehyde, fractions containing the inhibitor-labeled peptide fragment could be identified (panel D, Figure 4). Also, this peptide was shown (panel C, Figure 4) to produce strong fluorescence emission at 340 nm, indicating that it contains one or more tryptophan residues. When analyzed by mass spectrometry, this fragment was shown to have a MW of 1379.7 Da (Figure 5). Analysis of this MW using the GPMAW program (*18*) suggested that this fragment arose from covalent modification of peptide W310−K318 (MW 1102.4 Da) with the intermediate generated from the inhibitor, which results in an increase in MW of 277.4 Da. This peptide was also indicated by the following evidences: (i) it contains one tryptophan residue, which is consistent with fluorescence detection; and (ii) the sequence fragments in mass spectra of this inhibitor-modified peptide matched very well with the expected pattern according to the Roepstorff and Fohlman fragmentation (*19*) (Figure 5). Therefore, the nucleoside labeling site should be Lys318.

DISCUSSION

Parry et al. (*10*) described that the inactivation of AdoHcy hydrolase by 5′,5′,6′,6′-tetradehydro-6′-deoxyhomoAdo resulted in the homotetramer having two subunits in the NADH form, which were covalently modified by 2 equiv of the inhibitor. The other two subunits were devoid of cofactor but contained 2 equiv of the inhibitors covalently bound. These observations are consistent with a mechanism involving the 3′-oxidative activity of AdoHcy hydrolase, i.e., the acetylenic analogue of homoAdo is oxidized to the 3′-keto derivative, which rearranges to the allene. The electrophilic allene can then react with a protein nucleophile, resulting in the formation of a covalent adduct (Scheme 1.)

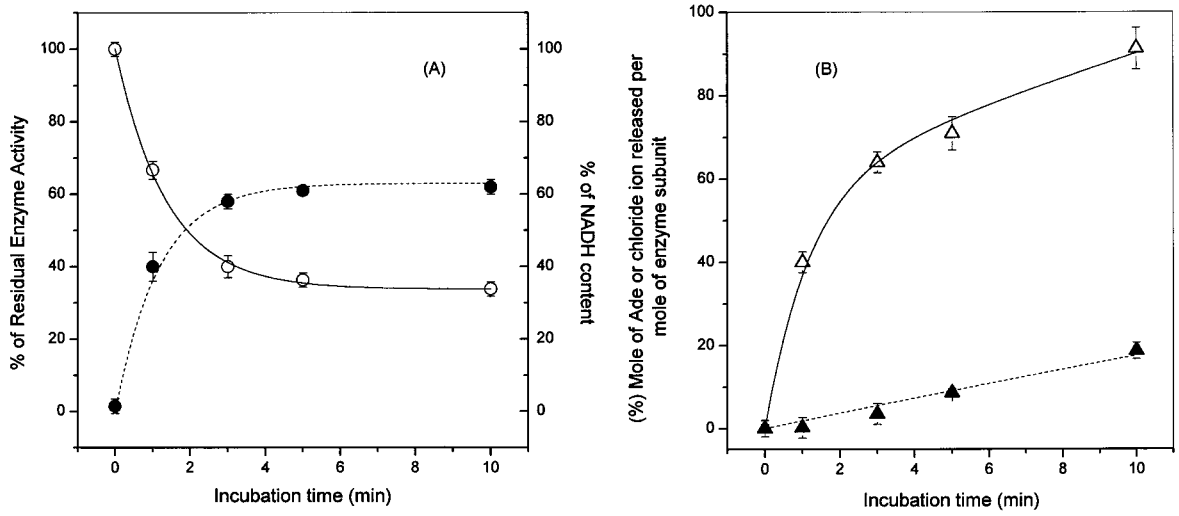


FIGURE 3: Time course of residual enzyme activity, formation of E-NADH, release of Ade, and release of chloride ion upon incubation of AdoHcy hydrolase with compound **a**. AdoHcy hydrolase (42 μM, NAD^+ form) was incubated with 100 μM 6′-chloro analogue in buffer A at 25 °C for the indicated times. Samples containing 2, 100, 200, and 100 μg of protein were withdrawn and used for assays of residual enzyme activity, NADH content, chloride ion release, and Ade release, respectively, as described in the Materials and Methods. (○) Solid line, % of residual enzyme activity; (●) dashed line, % of NADH content; (△) solid line, % ratio of released chloride ion to enzyme; (▲) dashed line, % ratio of released Ade to enzyme.

Table 2: Kinetic Parameters for Loss of Enzyme Activity, Formation of NADH, and Release of Ade and Chloride Ions from Compound **a**[a]

process	rate constant (k_{app}) (min^{-1})	*A* value (%)
loss of enzyme activity	0.85 ± 0.03	65.3 ± 0.9
formation of NADH	0.87 ± 0.05	61.4 ± 0.4
release of chloride ion	0.85 ± 0.09 (k_{app1})	61.7 ± 3.3 (A_1)
	0.015 ± 0.003 (k_{app2})	188 ± 11 (A_2)
release of Ade	0.014 ± 0.002	147 ± 20

[a] AdoHcy hydrolase (NAD^+ form, 2.0 mg/mL) was incubated with 100 μM compound **a** at 25 °C. An aliquot of the reaction mixture was withdrawn at various times and the reaction was terminated by either diluting 250-fold with 1 mM Ado (activity assay) or adding 3× vol of absolute EtOH (NADH content and chloride ion-assay) or 20 μL of 5 M $HClO_4$ (Ade assay). The assays were carried out as described in the Materials and Methods. The kinetic constants were calculated by fitting the data to either first order [$y = A(1 - e^{-k_{app1}t})$] or second-order [$y = A_1(1 - e^{-k_{app1}t}) + A_2 (1 - e^{-k_{app2}t})$] processes using a program Origin (OriginLab, Northampton, MA).

On the basis of the observation by Parry et al. (*10*), we prepared 6′-haloacetylenic analogues of homoAdo as potential substrates for the 3′-oxidative and/or 5′/6′-hydrolytic activity of the enzyme (*13*). If these 6′-haloacetylenic analogues of homoAdo are substrates of the enzyme's 3′-oxidative activity, they might inactivate AdoHcy hydrolase by the mechanism shown in Scheme 1. However, if they are substrates for the 5′/6′-hydrolytic activity, addition of water across the triple bond might generate acyl halides and/or α-halomethyl ketones at the active site of the enzyme (Scheme 2). These strong electrophiles might then be attacked by a protein nucleophile, resulting in covalent modification of AdoHcy hydrolase.

On the basis of the present experimental results, compound **c** appears to be a substrate only for the 3′-oxidative activity of the enzyme because no product of water addition at the triple bond (i.e., Ade, iodide ions) was observed. Hence, compound **c** probably inactivates AdoHcy hydrolase by a mechanism similar to that described by Parry et al. (*10*) for the acetylenic analogue of homoAdo (Scheme 1). The

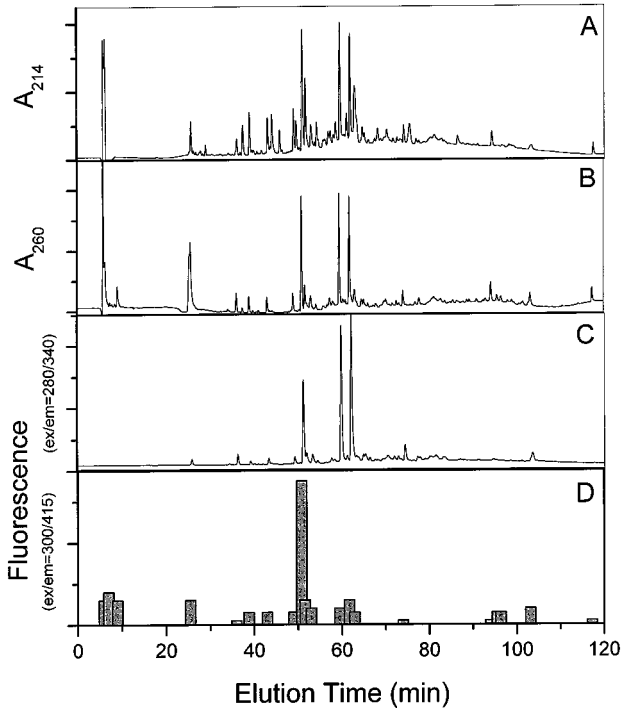


FIGURE 4: HPLC tryptic peptide map of inhibitor (compound **a**)-labeled AdoHcy hydrolase. The trypsin digested inhibitor−enzyme complex was applied to a C18 reversed-phase HPLC column (Vydac 218TP54, C18, 5 μM, 4.6 × 250 mm). Elution was carried out with a gradient of acetonitrile in 0.1% trifluoroacetic acid from 2 to 80% in 105 min. Eluted peptides were monitored at A_{260}, A_{214}, and 340 nm fluorescence emission with excitation at 280 nm. The bar graph below indicates the inhibitor content for each elution peak analyzed using the method of bromoacetaldehyde derivatization (see the Material and Methods).

experimental observations supporting this conclusion include (i) the enzyme is completely inactivated by compound **c**; (ii) upon complete inactivation of the enzyme, approximately 50% of the cofactor (NADH) is still bound to the enzyme and approximately 50% is released into the incubation mixture; and (iii) upon complete inactivation of the enzyme, approximately 0.9 mol of the inhibitor are

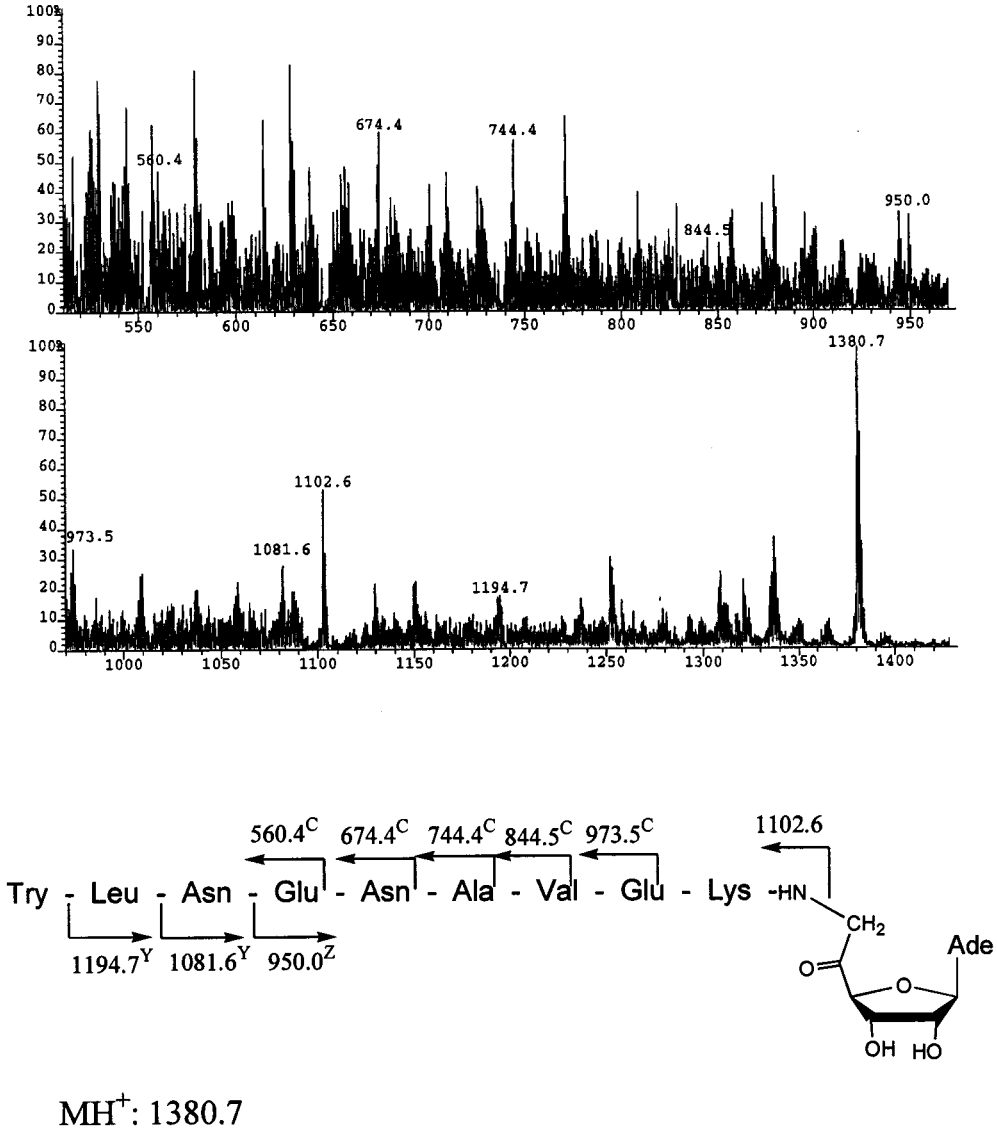
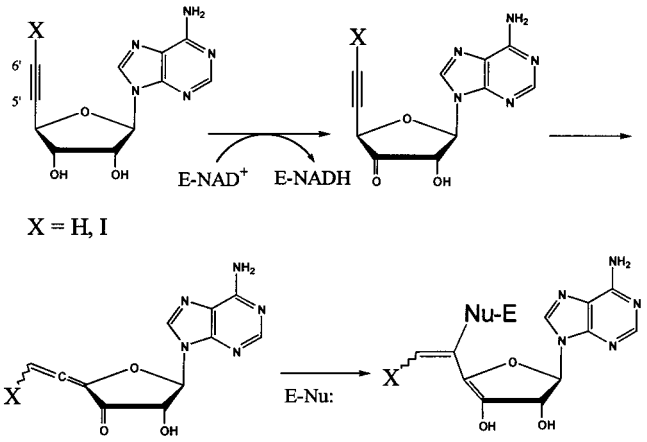


FIGURE 5: FAB mass spectrum of compound **a**-labeled tryptic peptide fragment of AdoHcy hydrolase. Inset: the deduced sequence of the inhibitor-labeled peptide with the identified fragment ions (C, Y, Z: N or C-terminal ions according to Roepstorff and Fohlman fragmentation nomenclature).

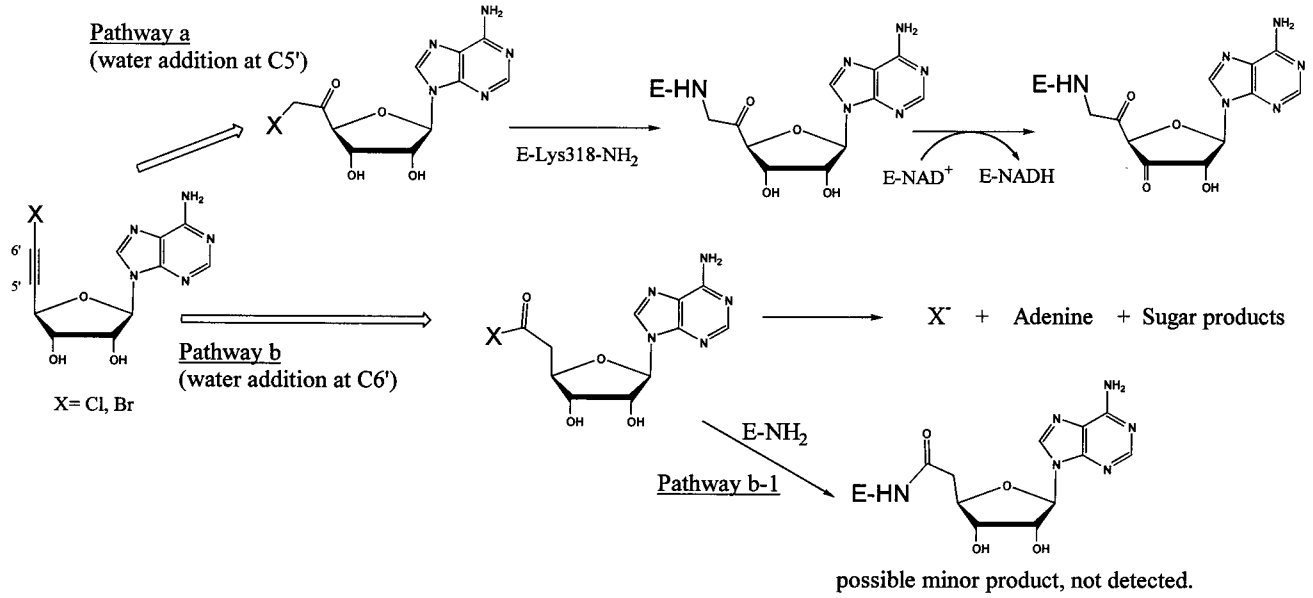
Scheme 1: Mechanisms of Inactivation of AdoHcy Hydrolase by 5′,5′,6′,6′-Tetradehydro-6′-deoxyhomoadenosine and Its 6′-Iodo Analogue



bound/mol of enzyme subunit. The inhibitor−enzyme adduct appears to be linked by a covalent bond that is stable to denaturation with 8 M urea under strong alkaline conditions (0.1 M LiOH) but unstable to denaturation under acidic or neutral conditions. These observations are consistent with the results reported by Parry et al. (*10*) for the acetylenic analogue of homoAdo. These data suggest involvement of the 3′-oxidative activity in the "activation" of the inhibitor, resulting in a type II mechanism of inactivation (Scheme 1).

Unfortunately, because of the instability of the inhibitor (compound **c**)−enzyme complex under near neutral denaturation conditions, we were unable to identify the specific amino acid residue that is covalently modified by this inhibitor. However, the crystal structure of an AdoHcy hydrolase (NADH-form)-3′-keto-DHCeA (*3*) shows that three nucleophilic amino acid residues are close to the 5′-position of the substrate, i.e., His55, His301 and Asp131. Any of these residues could be a possible candidate for the nucleophile attacking the electrophilic allene intermediates generated from the acetylenic analogue of homoAdo or compound **c** (Scheme 1) as suggested by Parry et al. (*10*). However, Asp adducts and His adducts of the type shown in Scheme 1 could be unstable under basic conditions (*20*).

Scheme 2: Mechanisms of Inactivation of AdoHcy Hydrolase by 6′-Chloro/Bromo-5′,5′,6′,6′-tetradehydro-6′-deoxyhomoadenosines



Therefore, we cannot rule out the possibility that the nucleophile is a Lys residue.

It is possible that compounds **a** and **b** might inactivate AdoHcy hydrolase by a similar mechanism due to their structural homology with compound **c**. However, our experimental results indicated that these inhibitors appear to be substrates for the 5′/6′-hydrolytic activities as well as the 3′-oxidative activity of the enzyme. Experimental observations supporting this conclusion include (i) the enzyme was only partially inactivated (approximately 50%) by compounds **a** or **b**; (ii) upon incubation of the enzyme with these inhibitors, only partial reduction of the E-NAD$^+$ to E-NADH was observed; (iii) upon incubation of the enzyme with these inhibitors, Ade and halide ions were generated in amounts suggestive of a process involving enzyme catalysis; and (iv) inactivation of the enzyme with compound **a** resulted in a total of 4 mol of inhibitor bound per mole of homotetramer. Two moles of inhibitor per mole of homotetramer was shown to be covalently bound to the enzyme. In this case, the covalent linkage was stable to the experimental conditions used to generate tryptic fragments allowing the use of mass spectrometry to identify Lys318 as the residue undergoing chemical modification. On the basis of crystal structures of AdoHcy hydrolases (*3*, *4*), Lys318 has been shown to be located near the substrate binding site (Figure 6); hence, it is a plausible nucleophile to react with the active intermediate generated from these inhibitors.

Therefore, key to the mechanism by which compounds **a** or **b** inactivate AdoHcy hydrolase is their substrate activity for the 5′/6′ hydrolytic activity of the enzyme. The experimental evidences described above are consistent with the mechanisms proposed in Scheme 2. Enzyme-catalyzed addition of water at the 5′-position of either inhibitor will generate α-halomethyl ketones, which are strong electrophiles. In contrast, addition of water at the 6′-position of either of these inhibitors will generate acyl halides, which are also strong electrophiles and could covalently modify the enzyme. Alternatively, these acyl halides can chemically degrade to Ade and halide ions as shown by Yuan et al. (*8*, *9*). By comparing the kinetics of enzyme inactivation with the kinetics of Ade and halide ion release upon incubation of AdoHcy hydrolase with compound **a**, the contributions of these two pathways could be determined. The results showed that loss of enzyme activity, NAD$^+$ depletion, and release of Ade are all pseudo-first-order processes, whereas the release of chloride ion follows second-order kinetics. The slow rate of chloride ion release, which is consistent with the rate of Ade release, should represent the 6′-hydrolytic activity (pathway b, Scheme 2). The fast rate of chloride ion release, which is consistent with the rate of enzyme inactivation and conversion of E-NAD$^+$ to E-NADH, should, therefore, represent the 5′-hydrolytic activity (pathway a, Scheme 2). The partition ratio between the 5′-hydrolytic

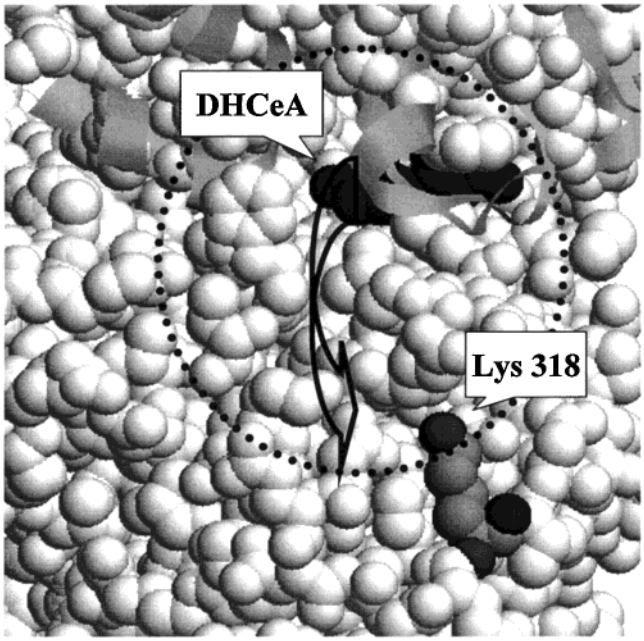


FIGURE 6: Illustration of the location of Lys318. The circle and the arrow indicate the substrate binding channel of AdoHcy hydrolase. The structural data are from the Protein Data Bank: 1a7a (*3*) and 1b3r (*4*).

activity (pathway a) and the 6′-hydrolytic activity (pathway b) is approximately 60:1. It is obvious that the 5′-hydrolytic activity should lead to half-of-site inactivation of the enzyme and modification of two subunits of this homotetramer. Although the acyl halide, which is the product of the 6′-hydrolytic activity, might also covalently modify the enzyme (pathway b-1, Scheme 2), its covalent modification product was not detected, probably due to instability of the active intermediate and much lower partition ratio (1:60) of 6′ to 5′ hydrolytic activity.

It is interesting to note that Yuan et al. (*11*) reported that the type II inhibitor BDDFHA modified Arg196 in human AdoHcy hydrolase rather than Lys318, which was shown in this paper to be modified by the acetylenic analogues **a** and **b**. BDDFHA was proposed to be primarily transformed by the 6′-hydrolytic activity of the enzyme to the acyl fluoride intermediate (*11*). The differences observed between the enzyme residues modified by BDDFHA and the acetylenic analogues **a** and **b** probably reflect the differences in the chemical reactivity and/or the juxtaposition of the α-halomethyl ketone (primary product arising from compound **a**) versus the acyl fluoride (primary product arising from BDDFHA) with the nucleophiles near the enzyme's active site.

The covalent inhibitor−enzyme adduct generated by pathway a appears to retain residual 6′-hydrolytic activity since formation of Ade continued until all of the inhibitor was consumed. This observation suggests that the two subunits that are not modified by the inhibitors must have undergone subtle conformational changes resulting in retention of their 3′-oxidative and 6′-hydrolytic activities but loss of their 5′-hydrolytic activity. These results are similar to data observed with BDDFHA (*11*).

To date, five compounds have been identified as type II mechanism-based inhibitors of AdoHcy hydrolase; i.e., the acetylenic analogue of homoAdo, the 6′-iodo (**c**), 6′-bromo (**b**), and 6′-chloro (**a**) acetylenic analogues of homoAdo and BDDFHA. Compounds **a** and **b** and BDDFHA have comparable half-of-site inactivation mechanisms, whereas the acetylenic analogue of homoAdo and compound **c** have mechanisms which involve total depletion of NAD^+. However, the latter mechanisms may again have a half-of-site component since the totally inactivated enzymes are devoid of cofactor on two of the subunits.

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