Mechanism of Inactivation of *S*-Adenosylhomocysteine Hydrolase by (*E*)-5′,6′-Didehydro-6′-deoxy-6′-halohomoadenosines†

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Received September 14, 1993; Revised Manuscript Received January 10, 1994⊛

ABSTRACT: *S*-Adenosylhomocysteine (AdoHcy) hydrolase is irreversibly inactivated by (*E*)-5′,6′-didehydro-6′-deoxy-6′-halohomoadenosines (EDDHHAs, halogen = I, Br, and Cl). The inactivation is concomitant with the reduction of the enzyme-bound NAD^+ (E·NAD^+) to NADH, the release of halide ion, and the formation of adenine (Ade) from the EDDHHAs. The mechanism of this inactivation involves two catalytic pathways. Pathway a involves a rapid addition of water to the 5′,6′-bond of EDDHHAs and elimination of halide ion, resulting in the formation of 6′-carboxaldehyde **1** which then degrades chemically, resulting in the formation of Ade. Alternatively, 6′-carboxaldehyde **1** can be oxidized by E·NAD^+ to form 3′-keto-6′-carboxaldehyde **3** and the NADH form (inactive) of the enzyme. Like 6′-carboxaldehyde **1**, the 3′-keto derivative **3** degrades chemically to form Ade. Pathway b involves the oxidation of EDDHHAs to 3′-keto-EDDHHAs **2** by E·NAD^+, as the first step, and the subsequent release of halide ion to form 3′-keto-6′-carboxaldehyde **3**. Evidence in support of these mechanisms includes the observations that incubation of EDDHHAs with AdoHcy hydrolase generated large molar excesses of halide ions and Ade, that Ade was shown to eliminate spontaneously from 6′-carboxaldehyde **1**, and that the more rapid the halide ion release ($Cl^- > Br^- > I^-$) from the EDDHHAs or the greater the partition ratios (nonlethal turnovers/lethal event), the lower the enzyme inactivation efficiency. These mechanisms are consistent with proposals for the inactivation of AdoHcy hydrolase by (*Z*)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (ZDDFA) [Yuan, C.-S., Yeh, J., Liu, S., & Borchardt, R. T. (1993) *J. Biol. Chem. 268*, 17030–17037], indicating that AdoHcy hydrolase possesses two catalytic activities (*i.e.*, hydrolytic activity and oxidative activity) that are functionally independent.

S-Adenosyl-L-homocysteine (AdoHcy[1]) hydrolase (EC 3.3.1.1) catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and L-homocysteine (Hcy) (De la Haba & Cantoni, 1959). In recent years, AdoHcy hydrolase has become an attractive target for the design of antiviral agents (De Clercq, 1987; Keller & Borchardt, 1988; Wolfe & Borchardt, 1991; Liu et al., 1992a,b, 1993) because of its important role in regulating biological methylation reactions critical for viral replication by controlling the intracellular levels of AdoHcy (Borchardt, 1980). Inhibition of this enzyme results in intracellular accumulation of AdoHcy and subsequent inhibition of all *S*-adenosylmethionine-dependent methylation reactions, including viral mRNA methylations (Pugh et al., 1978; Borchardt & Pugh, 1979; Keller & Borchardt, 1986; Ransohoff et al., 1987; Hasobe et al., 1989).

The mechanism by which AdoHcy hydrolase catalyzes the hydrolysis of AdoHcy to Ado and Hcy (or, in the opposite direction, the formation of AdoHcy from Ado and Hcy) has been elegantly elucidated (Palmer & Abeles, 1979). In the reaction catalyzed by AdoHcy hydrolase, the first step in the hydrolytic direction is the oxidation of the 3′-hydroxyl group of AdoHcy by enzyme-bound NAD^+ (E·NAD^+) followed by β-elimination of L-Hcy to give the 3′-keto-4′,5′-didehydro-5′-deoxyadenosine intermediate. Michael addition of water to this intermediate affords 3′-ketoadenosine, which is then reduced by enzyme-bound NADH (E·NADH) to Ado.

On the basis of the observation (Palmer & Abeles, 1979) that 4′,5′-didehydro-5′-deoxyadenosine is a substrate for AdoHcy hydrolase, McCarthy and co-workers (McCarthy et al., 1989; Mehdi et al., 1990; Jarvi et al., 1991) synthesized vinyl fluoride analogs of this nucleoside, *e.g.*, (*Z*)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (ZDDFA), as potential mechanism-based inhibitors of AdoHcy hydrolase. The mechanism of the enzyme inactivation by ZDDFA has been reported recently by our group (Yuan et al., 1993). This mechanism includes a rapid addition of water to the 4′,5′-positions of ZDDFA and elimination of fluoride ion, resulting in the formation of the Ado 5′-carboxaldehyde and its 4′-epimer, which are then oxidized in a slower step to the 3′-keto-5′-carboxaldehyde and its 4′-epimer by reduction of the E·NAD^+ to E·NADH. It was demonstrated that addition of water to the 4′,5′-positions of ZDDFA is an enzyme-catalyzed hydrolytic process and is independent of the enzyme-catalyzed oxidation of the 3′-hydroxyl group.

† This work was supported by grants from the United States Public Health Service (GM-29332) and the American Cancer Society (DHP-34).

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⊛ Abstract published in *Advance ACS Abstracts,* March 1, 1994.

[1] Abbreviations: EDDHHAs, (*E*)-5′,6′-didehydro-6′-deoxy-6′-halohomoadenosines; EDDIHA, (*E*)-5′,6′-didehydro-6′-deoxy-6′-iodohomoadenosine; EDDBrHA, (*E*)-5′,6′-didehydro-6′-deoxy-6′-bromohomoadenosine; EDDClHA, (*E*)-5′,6′-didehydro-6′-deoxy-6′-chlorohomoadenosine; ZDDFA, (*Z*)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine; Ade, adenine; AdoHcy, adenosyl-L-homocysteine; E·NAD^+, enzyme-bound NAD^+; E·NADH, enzyme-bound NADH; BSA, bovine serum albumin; 5′-CNAdo, 5′-deoxy-5′-cyanoadenosine; DIBAL-H, diisobutylaluminum hydride; THF, tetrahydrofuran; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

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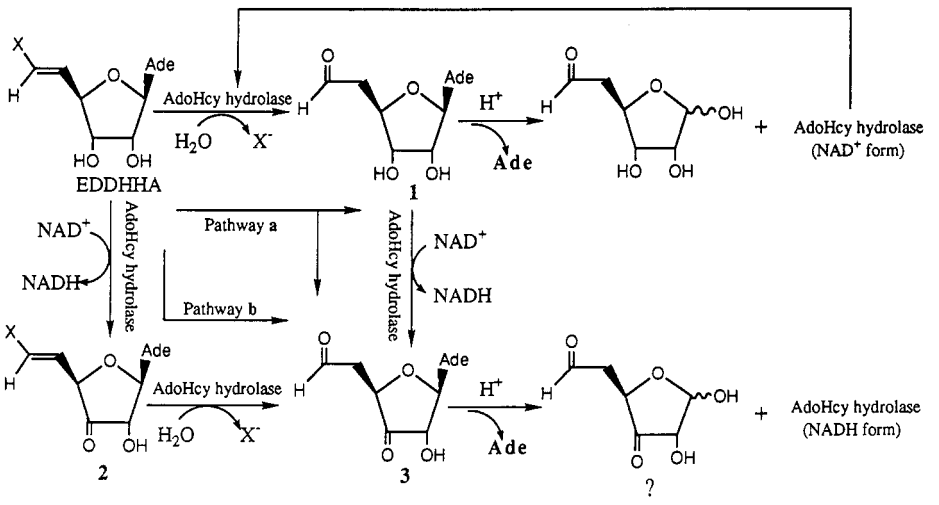
Scheme 1: Proposed Mechanism by Which EDDHHA Inactivates AdoHcy Hydrolase



compound	X
EDDIHA	I
EDDBrHA	Br
EDDClHA	Cl

Recently, a series of homovinyl halide analogs of ZDDFA was synthesized and shown to be potential mechanism-based inhibitors of AdoHcy hydrolase (Wnuk et al., 1994). In this study, we report the mechanism of AdoHcy hydrolase inactivation by (*E*)-5′,6′-didehydro-6′-deoxy-6′-halohomoadenosines (EDDHHAs, Scheme 1).

MATERIALS AND METHODS

Materials. AdoHcy, Ado, and Hcy were purchased from Sigma Chemical Company (St. Louis, MO). Standard iodide ion, bromide ion, and chloride ion were obtained from P. J. Cobert Associates, Inc. (St. Louis, MO). (*E*)-5′,6′-Didehydro-6′-deoxy-6′-iodohomoadenosine (EDDIHA), (*E*)-5′,6′-didehydro-6′-deoxy-6′-bromohomoadenosine (EDDBrHA), and (*E*)-5′,6′-didehydro-6′-deoxy-6′-chlorohomoadenosine (EDDClHA) were synthesized as described (Wnuk et al., 1994).

Purification of AdoHcy Hydrolase. The recombinant human placental AdoHcy hydrolase was purified from the cell-free extracts of *E. coli* transformed with the plasmid pPROKcd20 and grown in the presence of isopropyl β-thiogalactopyranoside by a procedure reported earlier (Yuan et al., 1993). The enzyme was purified by treatment of the cell-free extract with DEAE–cellulose, $(NH_4)_2SO_4$ fractionation (30–60%), and Sephacryl S-300 and DEAE–Sepharose column chromatographies. On SDS–PAGE, the purified enzyme showed a single band corresponding to a subunit M_r of 45 000. On size-exclusion chromatography, the enzyme had an M_r of 190 000. About 60 mg of homogeneous enzyme (0.9–1.1 units/mg) was obtained from a 1-L culture. For the experiments described in this article, the subunit M_r was used to calculate the molarity of enzyme solutions. The protein concentration was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Assay of AdoHcy Hydrolase Activity. AdoHcy hydrolase activity was assayed in the synthetic direction by measuring the rate of formation of AdoHcy from Ado and Hcy. The enzyme was incubated with 0.2 mM Ado and 5 mM Hcy in 500 μL of 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA (buffer A) at 37 °C for 5 min. The reaction was terminated by the addition of 25 μL of 5 N $HClO_4$. Insoluble material was removed by centrifugation, and the supernatant was analyzed for AdoHcy by HPLC using a C18 reversed-phase column (Econosphere C18, 5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL). The elution was carried out at a flow rate of 1 mL/min in two sequential linear gradients: 6–15% B in A over 0–9 min and 15–50% B in A over 9–15 min, where mobile phase A was 50 mM sodium phosphate buffer (pH 3.2) containing 10 mM 1-heptanesulfonic acid and B was acetonitrile. The peak of AdoHcy was monitored at 258 nm. The concentration of AdoHcy was determined by comparison of the peak area with that of a known quantity of authentic AdoHcy using a standard curve.

AdoHcy Hydrolase Inactivation Studies. The kinetic constants of enzyme inactivation were determined by the method of Kitz and Wilson (1962). Varying concentrations of inhibitor (60–800 nM) were incubated with AdoHcy hydrolase (10 nM) in buffer A at 37 °C for different amounts of time (0–8 min), and the activity remaining was determined in the synthetic direction as described above. Pseudo-first-order rate constants (K_{app}) were obtained from plots of log(% of activity remaining) vs incubation time, and K_i and k_{inact} values were estimated from the double-reciprocal plots of $1/K_{app}$ vs $1/[I]$ by using the following equation:

$$1/K_{app} = 1/k_{inact} + (K_i/k_{inact})/[I] \quad (1)$$

Determination of E·NAD⁺ and E·NADH. The quantitation of E·NAD^+ and E·NADH was performed by a fluorescence method as described earlier (Yuan et al., 1993). The kinetics of inhibitor-induced E·NADH formation was determined by measuring the increase in absorbance at 340 nm at different time intervals after the enzyme was mixed with inhibitors. AdoHcy hydrolase (5.4 mg) was dissolved in 2.8 mL of buffer A in a UV cuvette, and the temperature of the enzyme solution was maintained at 37 °C by a water bath. To the enzyme solution was added 200 μL of the inhibitor solution (12 mM) with mixing for 10 s. The UV spectrum (280–600 nm) was recorded periodically using an HP 8452 diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). The instrument was referenced against the enzyme solution with the addition of 200 μL of water instead of the inhibitor. The spectrum was recorded until no increase in the absorbance at 340 nm was observed (at 45 min). The first-order rate constant for the increase in absorbance at 340 nm was calculated by

fitting the data (absorbance at 340 nm at different times after mixing) to an exponential function, $A = A_{max}(1 - e^{-kt})$, using XYmath computer software (Sacramento, CA), where A is the absorbance at 340 nm at time t, A_{max} is the maximum absorbance at 340 nm observed, and k is the first-order rate constant of the increase in absorbance at 340 nm.

Determination of Halide Ion Release from EDDHHAs. Halide ions (I^-, Br^-, and Cl^-) released from EDDHHAs by incubation with AdoHcy hydrolase were determined by ion-exchange chromatography in an HPLC system equipped with a conductivity detector. AdoHcy hydrolase (2.7 mg) was dissolved in 2.2 mL of 5 mM phosphate buffer (pH 7.2), to which 200 μL of the inhibitor solution (9.6 mM) was added. The reaction mixture (25 μM enzyme and 800 μM inhibitor) was incubated at 37 °C for different time intervals (2.5–40 min). At each time point, an aliquot of the reaction mixture containing 270 μg of protein (240 μL) was withdrawn and added to a test tube containing 700 μL of 97% ethanol. The precipitated protein was removed by filtration through an Amicon Centricon-3 microconcentrator (3000 M_r cutoff), and the filtrate and washing were combined and lyophilized. The residue was dissolved in 100 μL of water, 30 μL of which was injected onto an ion-exchange column (Anion/R, 250 × 4.1 mm, 10 μm, Alltech, Deerfield, IL) in an HPLC system equipped with a conductivity detector (690 Ion Chromatograph, Omega Metrohm, Ltd., Herisau, Switzerland) for halide ion analysis. For iodide ion assay, 1.5 mM sodium octanesulfonate (pH unadjusted) was used as the mobile phase, and the ion was eluted isocratically at a flow rate of 1.5 mL/min. For bromide and chloride ions, the mobile phase was 1.5 mM *p*-hydroxybenzoic acid/2% methanol (pH 8.5, adjusted by LiOH). The elution was performed isocratically at a flow rate of 2 mL/min. Control experiments were performed using BSA and authentic halide ions to calculate the recovery from the above procedure. Recoveries of 69–72% were obtained in the control experiments. Quantitation of halide ions was achieved by comparison of the ion peak area with that of a known quantity of authentic ions using a standard curve. The first-order rate constants for halide ion release from the EDDHHAs were obtained by fitting the data to an exponential function.

Determination of Ade Formation. AdoHcy hydrolase (2 nmol) was incubated with EDDHHAs (75 nmol) in 0.25 mL of buffer A at 37 °C, and the reaction was stopped at different time intervals by the addition of 10 μL of 5 N $HClO_4$. The precipitate was removed by centrifugation, and the supernatant was analyzed by HPLC using a reversed-phase C18 column (Econosphere, Alltech, 250 × 4.6 mm, 5 μm, Deerfield, IL). Ade was separated from EDDHHAs by three sequential linear gradients: 6–25% B in A over 15 min, 25–40% B in A over 15–20 min, and 6% B in A over 20–30 min, where A was 50 mM sodium phosphate buffer (pH 3.2) containing 10 mM 1-heptanesulfonic acid and B was acetonitrile. The chromatography was monitored by UV at 258 nm. Quantitation of the Ade formed was carried out using a calibration curve of authentic Ade. The first-order rate constants for Ade formation from EDDHHAs were obtained by fitting the data to an exponential function.

The isolated Ade was identified by coinjection with authentic Ade on the HPLC system. Further characterization was performed by chemical ionization (CI) mass spectral analysis on a Nermag (Paris, France) R10-10 quadrupole GC/MS system. The sample was evaporated from a direct insertion probe with ammonia as the reagent gas.

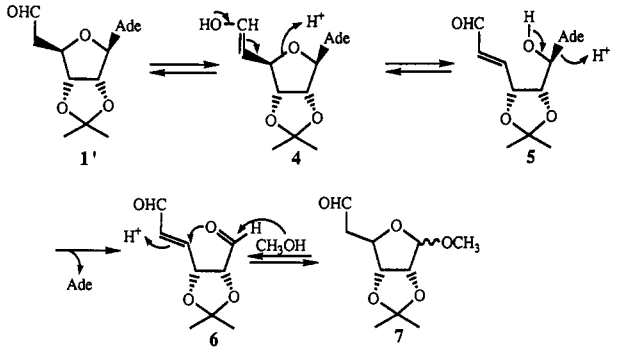
To determine whether Ade formation was the result of the acidic conditions used to denature the enzyme and employed in the HPLC system, an analogous experiment was carried out in which the enzyme was denatured with 96% ethanol (final ethanol concentration 60%), and a neutral HPLC mobile phase (5 mM phosphate buffer, pH 6.5) was used.

To determine whether the Ade formed by the reaction of AdoHcy hydrolase and EDDHHAs was enzyme-bound, three samples (0.5 mL each) containing 22 nmol of enzyme and 120 nmol of EDDIHA were incubated at 37 °C for 90 min, so that all of the EDDIHA in the mixture was reacted. One of the samples was filtered through an Amicon Centricon-3 microconcentrator, and the retentate was washed once with water (200 μL). The combined filtrate and washing were examined for Ade concentration. Another sample was acidified to pH 2.5 with 5 N $HClO_4$ to denature the enzyme, and the Ade concentration in the supernatant was examined. The last sample was dialyzed overnight against buffer A before denaturation of the protein with $HClO_4$. The supernatant from centrifugation was examined for Ade. Ade concentrations in the three samples were compared.

Determination of Partition Ratio. The partition ratios of EDDHHAs were determined kinetically by measuring the residual enzyme activity and the product (Ade) generated. AdoHcy hydrolase (20 nmol) was incubated with 800 nmol of EDDHHAs at 37 °C for various time intervals. At each time point, two samples, one containing 2 nmol and one 20 pmol of enzyme, were withdrawn and examined for Ade formation and residual activity, respectively. The sample for Ade assay was immediately mixed with 25 μL of 5 N $HClO_4$, and the supernatant from centrifugation was injected onto the C18 reversed-phase column under the same conditions as described above. The sample for enzyme activity assay was immediately diluted with 1 mL of buffer A (400-fold dilution), frozen in an acetone–dry ice bath, and then transferred to an ice–water bath to thaw prior to activity assay by HPLC. The ratio of moles of Ade generated to moles of the enzyme inactivated was expressed as the partition ratio.

Synthesis of the 6′-Carboxaldehyde of Homoadenosine. Synthesis of the 2′,3′-*O*-isopropylidenehomoadenosine-6′-carboxaldehyde **1′** (Scheme 2) was carried out by reduction of 2′,3′-*O*-isopropylidene-5′-deoxy-5′-cyanoadenosine (5′-CNAdo), which was synthesized according to published procedures (Sakami, 1961; Cullen et al., 1984; Meyer & Follman, 1980), with diisobutylaluminum hydride (DIBAL-H) as the reducing agent (Little et al., 1982). 2′,3′-*O*-Isopropylidene-5′-CNAdo (200 mg, 0.95 mmol) was dissolved in dried tetrahydrofuran (THF) (5 mL), and the solution was cooled to −78 °C in a dry ice–acetone bath. DIBAL-H (1 M, 2.9 mL) was then added over 5 min, and the solution was stirred at −78 °C for 30 min, then warmed to room temperature, and stirred until TLC showed that the starting material was completely consumed. After the reaction mixture was cooled to −78 °C, methanol (0.5 mL) was added to quench the reaction. After the reaction mixture was warmed to 0 °C, chloroform (5 mL) was added following the addition of hydrochloric acid solution (0.5 M, 3.5 mL). The solution then was stirred at 0 °C for 1.5 h, and the precipitate was filtered and washed with $CHCl_3$. The filtrate was evaporated and separated through preparative TLC using 10% MeOH in $CHCl_3$ as solvent. Only the decomposition products, Ade and the ribosyl moiety **7** (Scheme 2), were recovered. Structures of these degradation products were confirmed by ^1H NMR analysis. Ade: δ (CD_3OD, ppm) 8.19 (s, 1 H), 8.13 (s, 1 H). Compound **7**: δ ($CDCl_3$, ppm) 9.83 (s, 1 H),

Scheme 2: Proposed Mechanism for the Decomposition of 1'



4.88 (s, 1 H), 4.75 (dd, J_1 = 5.8 Hz, J_2 = 3.7 Hz, 1 H), 4.57 (d, 5.8 Hz, 1 H), 4.38 (m, 1 H), 3.32 (s, 3 H), 2.87 (dd, J_1 = 6.4 Hz, J_2 = 1.4 Hz, 2 H), 1.44 (s, 3 H), 1.30 (s, 3 H).

RESULTS

Inactivation of AdoHcy Hydrolase by EDDHHAs. When the purified AdoHcy hydrolase was incubated with a large molar excess of EDDHHAs (*e.g.*, EDDIHA), a time-dependent loss of activity was observed as shown in Figure 1. The inactivation displayed pseudo-first-order kinetics in residual enzyme activity and saturation kinetics in inhibitor concentration. The potencies of the inhibitory activity of EDDHHAs toward AdoHcy hydrolase were observed to be EDDIHA > EDDBrHA > EDDClHA. As noted in Table 1, the three EDDHHAs showed similar K_i values (96–134 nM) but quite different k_{inact} values (0.058–0.015 min^{-1}). The half-time ($t_{1/2}$) for EDDIHA-induced loss of activity at 100 nM concentration was 25.7 ± 1.2 min. An identical concentration of EDDBrHA and EDDClHA gave half-times of 53.3 ± 2.7 and 77.2 ± 4.6 min, respectively (Table 1). The inactivation of AdoHcy hydrolase by EDDHHAs was irreversible, as judged by the failure to regain catalytic activity after gel filtration to remove excess EDDHHAs or dialysis against buffer A.

Effect of EDDHHAs on E·NAD$^+$. Inactivation of AdoHcy hydrolase by EDDHHAs was accompanied by the reduction of E·NAD$^+$ to NADH, which was quantitatively determined by a fluorescence method. For example, a stoichiometry of 0.89 mol of NADH/mol of enzyme subunit was obtained when the enzyme (2.5 nmol) was completely inactivated by EDDIHA (50 nmol) after incubation at 37 °C for 1 h. This stoichiometric reduction of E·NAD$^+$ also indicated that the number of binding sites was approximately 1 per enzyme subunit. The kinetics of the E·NAD$^+$ reduction by EDDIHA was carried out by observing the increase in absorbance at 340 nm when EDDIHA was mixed with the enzyme, which was presumed to be due to the formation of NADH. A time-dependent increase in absorbance at 340 nm was observed (Figure 2). It reached a maximal change after 45 min of incubation, which corresponds to 97% inactivation of the enzyme. A first-order rate constant of 0.06 min^{-1} was obtained at the saturating inhibitor concentration (800 μM, analogous to k_{inact}) from the plot of the absorbance at 340 nm vs time (Figure 2, inset). The inactivated enzyme, after removal of the excess inhibitor by dialysis, could not be reactivated by incubation with 2 mM NAD$^+$ at 37 °C for 1 h.

Release of Halide Ion from EDDHHAs. Incubation of AdoHcy hydrolase with EDDHHAs resulted in the release of halide ion. As shown in Figure 3, when a large molar excess of EDDIHA was incubated with AdoHcy hydrolase, iodide ion (retention time, 3.4 min) was observed on the ion-exchange chromatogram, with a maximum of 7.3 ± 0.2 mol of iodide ion/mol of enzyme subunit. The first-order rate constant for iodide ion release was estimated to be 0.33 min^{-1} (Figure 3, inset). Incubation of EDDIHA alone or with BSA did not result in the release of iodide ion (data not shown). Release of bromide ion and chloride ion from EDDBrHA and EDDClHA upon incubation with AdoHcy hydrolase was also observed. The rates of the enzyme-catalyzed release of halide ion from EDDHHAs were in the order, EDDClHA > EDDBrHA > EDDIHA.

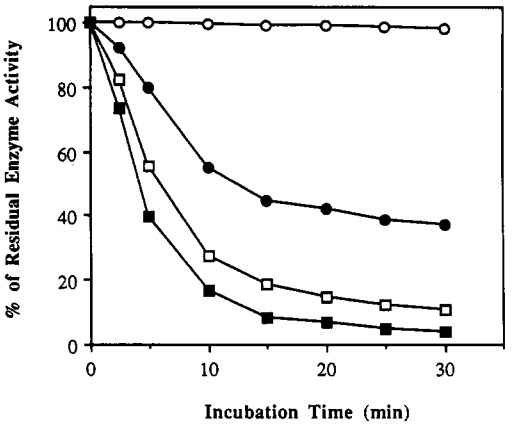


FIGURE 1: Time-dependent inactivation of AdoHcy hydrolase by EDDIHA. AdoHcy hydrolase was incubated with EDDIHA (○, control; ●, 10 μM; □, 25 μM; ■, 50 μM) in buffer A at 37 °C for various times. At the indicated times, residual enzyme activity was determined in the synthetic direction using Ado and Hcy as substrates, as described in Materials and Methods.

Ade Formation. When AdoHcy hydrolase was incubated with EDDHHAs, Ade formation was observed. For example, Figure 4 is an HPLC chromatogram from a reaction mixture of AdoHcy hydrolase and EDDIHA. Two main peaks appeared on the chromatogram. The peak with retention time 18.0 min was the unreacted EDDIHA, and the peak with retention time 11.1 min was Ade, which had the same retention time as authentic Ade when examined by coinjection on HPLC (Figure 4, inset). A small peak with a retention time of approximately 15 min was also observed, but its structure was not characterized. When the Ade peak was collected and subjected to chemical ionization (CI) mass spectrometric analysis, an ion (MH^+) at m/z 136 was obtained, which is consistent with the structure of Ade. Ade was also formed when the enzyme was incubated with EDDBrHA or EDDClHA (data not shown). The first-order rate constant of Ade release from EDDIHA at 37 °C, pH 7.2, was estimated to be 0.31 min^{-1} by fitting the data to an equation for a first-order process. When the enzyme was completely inactivated by EDDIHA, 7.5 ± 0.1 mol of Ade was formed per mole of enzyme subunit.

Incubation of EDDHHAs in buffer A alone or with an inert protein such as BSA followed by the same treatment and HPLC procedures did not result in the formation of Ade (data not shown). When the enzyme inactivated by EDDHHAs was denatured by treatment with ethanol, and the supernatant was analyzed in a neutral HPLC mobile phase, Ade was formed (data not shown). These results show that Ade was not formed by acid hydrolysis in the enzyme denaturation or the acidic HPLC mobile phase.

The amount of Ade in the filtrate from the undenatured reaction mixture of the enzyme and EDDIHA was compared with that in the supernatant of the reaction mixture after denaturation of the enzyme. No difference in the amount of Ade was observed between the two samples. In fact, Ade equivalent to 95% of the initially added EDDIHA was

Table 1: Kinetic Constants and Partition Ratios of EDDHHAs toward Recombinant Human Placental AdoHcy Hydrolase[a]

inhibitor	K_i (nM)	k_{inact} (min^{-1})	k_{inact}/K_i (M^{-1} min^{-1})	$t_{1/2}$ (min)[b]	partition ratio[c]
EDDIHA	96 ± 7.6	0.058 ± 0.007	6.04×10^5	25.7 ± 1.2	6.5 ● 0.10
EDDBrHA	134 ± 6.4	0.037 ± 0.004	2.76×10^5	53.3 ● 2.7	11.5 ± 0.45
EDDClHA	110 ± 8.2	0.015 ± 0.004	1.36×10^5	77.2 ± 4.6	28.2 ± 1.20

[a] Data are the average of two measurements. [b] The half-time ($t_{1/2}$) of enzyme inactivation at 100 nM EDDHHA. [c] Ade formation was used to measure turnover.

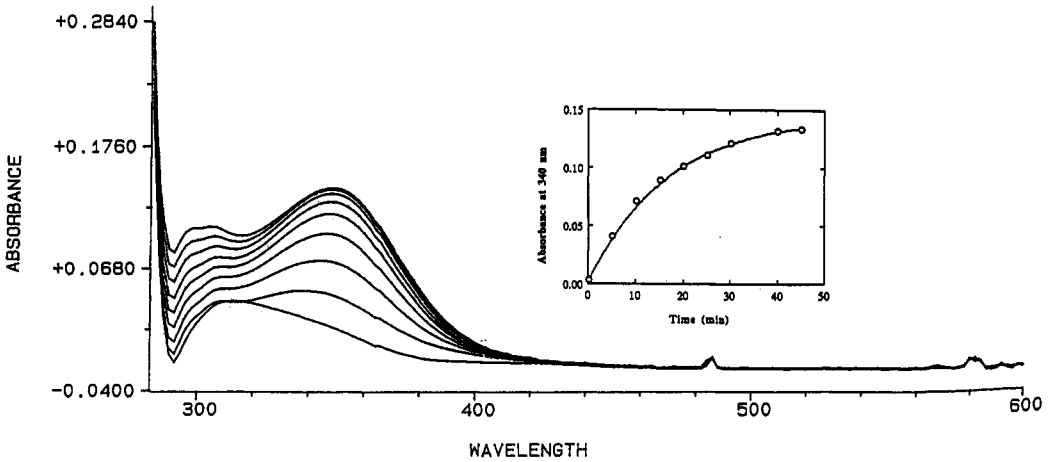


FIGURE 2: Spectra of a mixture of AdoHcy hydrolase and EDDIHA. AdoHcy hydrolase (40 μM) in buffer A was mixed with EDDIHA (800 μM) in a UV cuvette and incubated at 37 °C for various times. At each time point (0.25, 5, 10, 15, 20, 25, 30, 40, and 45 min), the spectra were recorded. Inset: Plot of absorbance at 340 nm vs time. The curve is the fit of the data to an equation for a first-order process.

recovered from both samples. Furthermore, after dialysis followed by denaturation of the enzyme, no Ade was detected in the supernatant. Therefore, the Ade observed was simply released in the solvent and was not the original enzyme-bound compound. This was in agreement with the fact that Ade is a reversible inhibitor of rat liver AdoHcy hydrolase or recombinant human placental AdoHcy hydrolase and is not tightly bound to the enzyme (Takata & Fujioka, 1984; Yuan et al., unpublished data).

Partition Ratio. For irreversible mechanism-based inhibitors, an important indicator of potency is the partition ratio, or the number of turnovers per inactivation event. The partition ratio values for EDDHHAs toward AdoHcy hydrolase were determined kinetically by measuring the residual enzyme activity and the amount of Ade generated. The results for EDDIHA are shown in Figure 5. Since part of the total released Ade was from the contribution of the enzyme inactivation event (because Ade was not enzyme-bound), Ade equal to the amount of the inactivated enzyme was subtracted from the total amount of Ade released at each time point when the percent of inactivation was plotted against the amount of Ade produced (Figure 5, inset). At 100% inactivation of the enzyme (2 nmol), 13.2 nmol of Ade was produced, which gives a partition ratio of 6.6. The partition ratio values for EDDBrHA and EDDClHA were determined to be 11.5 ± 0.45 and 28.2 ± 1.2, respectively. As noted in Table 1, the smaller partition ratio value corresponds to the more potent (*i.e.*, k_{inact}/K_i) inhibitor.

Preparation and Degradation of the 6′-Carboxaldehyde **1′**. 2′,3′-*O*-Isopropylidene-5′-CNAdo was reduced to its 5′-imine analog using DIBAL-H as the reducing agent. It was then hydrolyzed with 0.5 N HCl at 0 °C as described in Materials and Methods. When the reaction mixture was extracted with $CHCl_3$, only Ade was observed in the aqueous phase by HPLC analysis. The crude residue from the organic phase after evaporation and drying was shown to contain the desired product, 6′-carboxaldehyde **1′**, when examined by ^1H NMR. However, when purification of the crude residue by TLC using 10% CH_3OH in $CHCl_3$ as the solvent was attempted, 6′-carboxaldehyde **1′** was not obtained. Instead, the products isolated were Ade and the 6′-carboxaldehyde sugar moiety **7** (Scheme 2). Various hydrolysis conditions with different acids (*e.g.*, $CF_3CO_2H:H_2O$ = 9:1 and $CF_3CO_2H:H_2O$:acetone = 1:2:17) gave only the degradation products in all cases. The glycosidic linkages of Ado and 2′,3′-*O*-isopropylidene-Ado were stable under the hydrolysis conditions described above (data not shown).

DISCUSSION

The inactivation of AdoHcy hydrolase by EDDHHAs was observed to be a time-dependent and concentration-dependent process. The inactivation appeared to be irreversible, since the enzyme activity could not be recovered after prolonged dialysis. During the course of the inactivation, E·NAD^+ was stoichiometrically reduced to E·NADH, and large molar excesses of halide ions and Ade were released into the solvent. Since no direct evidence was obtained concerning the fate of the 3′-keto-6′-carboxaldehyde sugar moiety derived from the EDDHHAs, whether the EDDHHAs are type I or type II mechanism-based inhibitors of AdoHcy hydrolase cannot be verified (Wolfe & Borchardt, 1991; Liu et al., 1992a). If the 3′-keto-6′-carboxaldehyde sugar does not covalently bind to the enzyme, the inactivation meets the criteria for a type I mechanism-based inhibitor of AdoHcy hydrolase. A type I mechanism-based inhibitor inactivates the enzyme by reducing E·NAD^+ to E·NADH in an irreversible manner, but it does not become covalently bound to the enzyme. In contrast, if the 3′-keto-6′-carboxaldehyde sugar becomes covalently bound to the enzyme, then the inactivation meets the criteria for a type II mechanism-based inhibitor of AdoHcy hydrolase. This type of inhibitor is envisioned to function by reduction of E·NAD^+ to E·NADH and generation of a chemically reactive intermediate at the enzyme active site, which could then react with a protein nucleophile to form a covalent adduct with the enzyme. To date, a type II mechanism-based inhibitor of AdoHcy hydrolase has not been identified. In the case of the

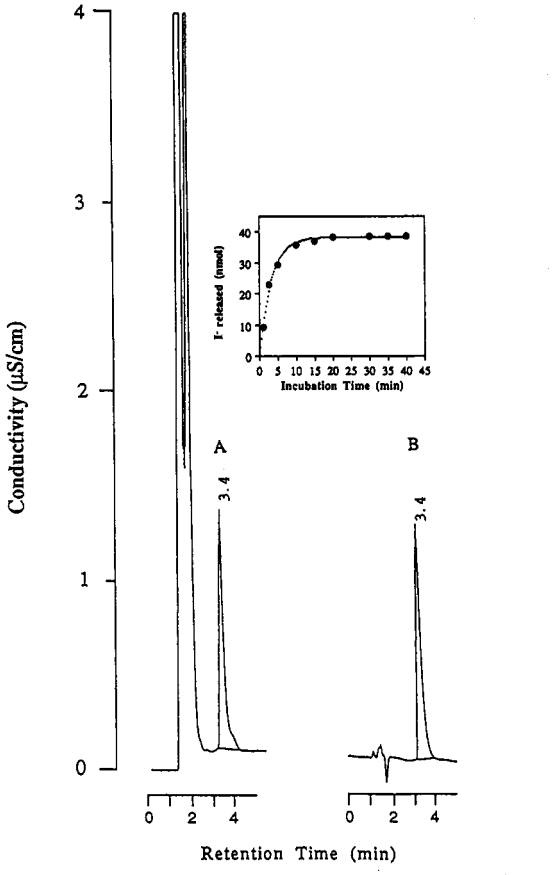


FIGURE 3: Ion-exchange chromatogram of iodide ion released from EDDIHA upon incubation with AdoHcy hydrolase. A reaction mixture (0.24 mL) consisting of AdoHcy (25 μM) and EDDIHA (800 μM) was incubated in 5 mM potassium phosphate buffer, pH 7.2, at 37 °C for 10 min, and the reaction was terminated by the addition of 700 μL of 97% ethanol, as described in Materials and Methods. The released iodide ion was separated by an ion-exchange column in an HPLC system equipped with a conductivity detector, using 1.5 mM sodium octanesulfonic acid as the mobile phase with a flow rate of 1.5 mL/min. (A) Iodide ion released from EDDIHA. (B) Authentic iodide ion. Inset: Plot of iodide ion released vs incubation time. The curve is the fit of the data to an equation for a first-order process.

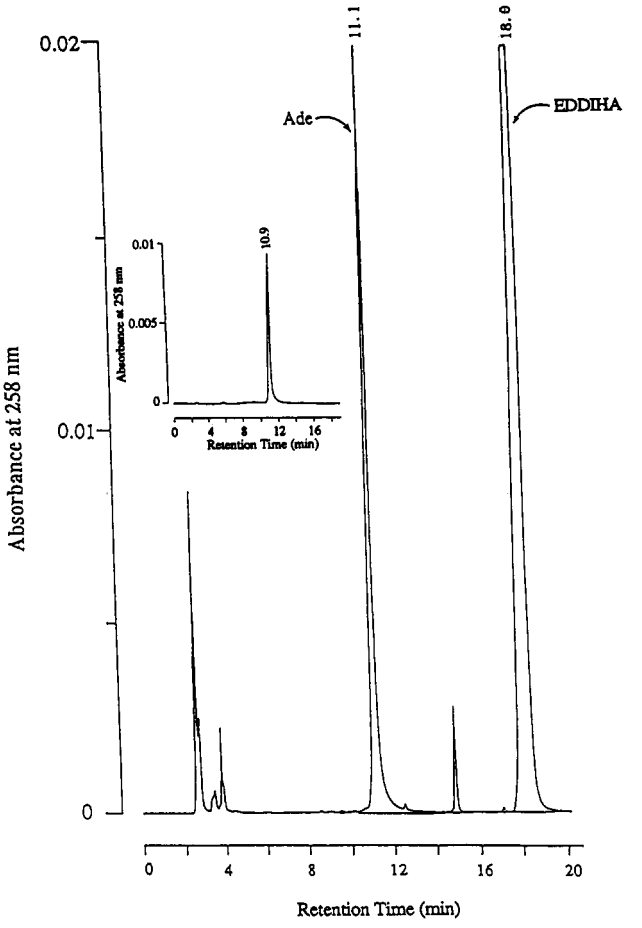


FIGURE 4: HPLC chromatogram of the products generated upon incubation of AdoHcy hydrolase and EDDIHA. AdoHcy hydrolase (2 nmol) and EDDIHA (75 nmol) were incubated in 0.25 mL of buffer A for 30 min. The reaction was stopped by the addition of 10 μL of 5 N $HClO_4$, and the supernatant from centrifugation was analyzed by HPLC, as described in Materials and Methods. Inset: Coinjection of the compound collected from the peak with a retention time of 11.1 min and authentic Ade.

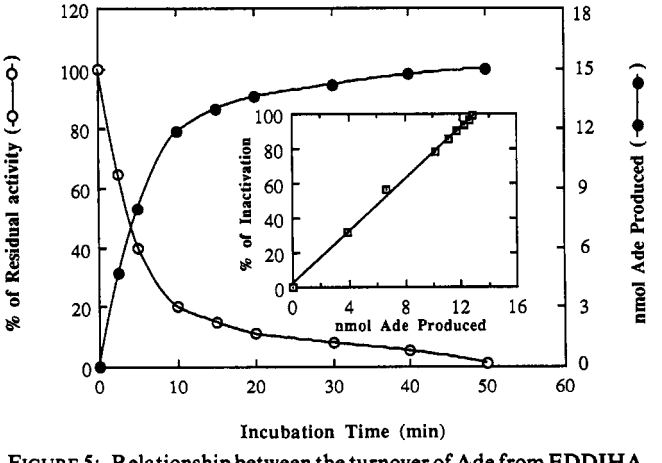


FIGURE 5: Relationship between the turnover of Ade from EDDIHA and the inactivation of AdoHcy hydrolase. AdoHcy hydrolase (2 nmol) was incubated with EDDIHA (75 nmol) in buffer A at 37 °C for various times. At each time point, as indicated, the residual activity and Ade formed were assayed as described under Materials and Methods. The partition ratio of EDDIHA is given as the number of turnovers divided by the inactivation events, which was obtained from the plot of % enzyme inactivation vs Ade (nmol) produced (inset).

EDDHHAs described in this article, it is not possible with the data available to determine whether these are type I or type II mechanism-based inhibitors.

Scheme 1 represents the proposed mechanisms by which EDDHHAs inactivate AdoHcy hydrolase. Two different pathways (a and b) appear to be involved in the inactivation of AdoHcy hydrolase by EDDHHAs. In pathway a, the first step of the inactivation is the rapid addition of water to the 5′,6′-bond of the EDDHHAs and elimination of halide ion, resulting in the formation of the 6′-carboxaldehyde **1**. In our previous study on the mechanism of enzyme inactivation by ZDDFA (Yuan et al., 1993), it was postulated that protonation of the ribose oxygen would facilitate nucleophilic attack by an enzyme-sequestered water molecule on the double bond, resulting in the elimination of fluoride ion and formation of the 5′-carboxaldehyde. In the case of EDDHHAs, while a similar mechansim is possible, the insertion of another carbon between the oxygen and the double bond would certainly attenuate the inductive effect of the protonated oxygen on the reactivity of the olefinic carbon toward nucleophilic attack by a water molecule. 6′-Carboxaldehyde **1** then partitions in two ways: (a) spontaneous chemical degradation of **1** to form Ade, or (b) oxidation at the 3′-hydroxyl group of **1** (a slow step) by reduction of E·NAD^+ to E·NADH to form 3′-keto-6′-carboxaldehyde **3**, which spontaneously degrades to Ade. In contrast, the first step of the enzyme inactivation in pathway b is oxidation of the 3′-hydroxyl group of the EDDHHAs

with reduction of E·NAD$^+$ to form 3′-keto-EDDHHAs **2**, which then react with enzyme-sequestered water at the 5′,6′-bond, resulting in the elimination of halide ion and the formation of 3′-keto-6′-carboxaldehyde **3**. The 3′-keto-6′-carboxaldehyde **3** can then degrade chemically to form Ade.

Evidence that is consistent with pathway a includes the observation that incubation of AdoHcy hydrolase with EDDHHAs generated large molar excesses of halide ion and Ade (see Table 1, partition ratios). The first-order rate of halide ion release (*e.g.*, 0.33 min^{-1} for EDDIHA) is much greater than the rate of E·NADH formation (0.06 min^{-1}) or the rate of enzyme inactivation (0.058 min^{-1}). Since the 6′-carboxaldehyde **1** is formed in the enzyme active site, it is highly possible that part of **1** is oxidized to **3** before depurination. However, it is clear from the partition ratios of the EDDHHAs that the major portion of Ade must be generated through pathway a without involving C-3′ oxidation. Evidence in support of the existence of pathway b comes from the observation that the more rapid the halide ion release ($Cl^- > Br^- > I^-$) from the EDDHHAs or the greater the partition ratio, the lower the inactivation efficiency. This strongly suggests that the major pathway by which the enzyme is inactivated is not pathway a but pathway b, through which every catalytic reaction results in a lethal event. In fact, pathway a may represent the main hydrolytic activity of the enzyme, which catalyzes the addition of water at the 5′,6′-bond of the EDDHHAs and elimination of halide ion, whereas pathway b may represent the main oxidative activity of the enzyme, which catalyzes oxidation at the 3′-hydroxyl group of EDDHHA and, in turn, causes the irreversible conversion of E·NAD$^+$ to E·NADH.

To verify the formation of 6′-carboxaldehyde **1** and 3′-keto-6′-carboxaldehyde **3** as intermediates in the mechanism of inactivation induced by EDDHHAs, efforts were made to synthesize 6′-carboxaldehyde **1** and study its propensity to undergo spontaneous depurination. Initially, attempts were made to prepare 6′-carboxaldehyde **1′**, which is a 2′,3′-*O*-protected form of **1**. However, generation of **1′** from 2′,3′-*O*-isopropylidene-5′-CNAdo resulted in formation of Ade and the 6′-carboxaldehyde sugar **7** (structures confirmed by ^{1}H NMR). Failure to isolate 6′-carboxaldehyde **1**, coupled with the fact that Ado and Ado-5′-carboxaldehyde are stable under the same hydrolysis conditions, indicates that the 6′-carboxaldehyde function causes new instability in the glycosidic linkage. A possible mechanism for Ade elimination from 6′-carboxaldehyde **1′** is proposed in Scheme 2. The ribosyl ring is opened by a retro-Michael addition process to form intermediate **5**, which upon release of Ade affords **6**. The nucleophilic addition of methanol or water to the 1-aldehyde function of **6** followed by intramolecular Michael addition gives the 6′-carboxaldehyde sugar **7**. The ring opening could be facilitated by the presence of either acid or base (Borchardt, 1979). The propensity of the 6′-carboxaldehyde **1** to depurinate greatly decreases the efficiency of the enzyme inactivation by EDDHHAs.

Abeles and co-workers (Abeles et al., 1980, 1982) reported that incubation of calf liver AdoHcy hydrolase with 2′-deoxyadenosine (2′-dAdo) resulted in the formation of Ade and that the eliminated Ade was tightly bound to the enzyme. The mechanism of Ade elimination was proposed to involve oxidation of 2′-dAdo at C-3′ by E·NAD$^+$, subsequent proton abstraction at C-2′, and elimination of Ade. We were surprised at the reported observation that the Ade eliminated from 2′-dAdo was tightly bound to the enzyme. However, careful reading of the experimental results of Abeles et al. (1982) suggested that their observation could have been an artifact of the workup procedure. According to their experimental procedure, Ade was determined only after denaturation of the enzyme. Thus, the possibility that elimination of Ade from 2′-dAdo occurred under the enzyme denaturation conditions could not be excluded. Therefore, the observed tight binding might not have been Ade, but 3′-keto-2′-dAdo, which would be expected to be trapped by the NADH form of the enzyme and undergo depurination after denaturation of the enzyme. The difference in Ade binding observed with 2′-dAdo and the EDDHHAs may also be due to the different enzyme sources used. Abeles and co-workers (1982) noted that the calf liver AdoHcy hydrolase used in their experiments might consist of two nonequivalent pairs of subunits, but only one subunit pair participated in catalysis. All four subunits probably bound 2′-dAdo, and Ade might have been bound to two of the subunits that did not participate in catalysis. This nonequivalence of enzyme subunits is not observed in our recombinant human placental AdoHcy hydrolase. All four subunits are identical and all participate in catalysis, which is supported by the stoichiometric reduction of appproximately 1 mol of E·NAD$^+$/mol of enzyme subunit.

The data presented here indicate that two catalytic activities, hydrolytic activity and oxidative activity, are involved in the enzyme inactivation by EDDHHAs. The two catalytic activities function independently in the course of the enzyme inactivation. These results support our earlier proposed mechanism of inactivation of AdoHcy hydrolase by ZDDFA (Yuan et al., 1993). It would be interesting to know whether the same functional groups are involved in catalyzing the addition of H_2O to the 5′,6′-bond of EDDHHAs, the 4′,5′-bond of ZDDFA, and the 4′,5′-bond of 3′-keto-4′,5′-didehydro-5′-deoxyadenosine. Further studies to clarify individual amino acid residues participating in the catalysis of the hydrolytic and oxidative reactions are being conducted in our laboratory using techniques of affinity labeling and site-directed mutagenesis.

ACKNOWLEDGMENT

The authors express their thanks to Dr. Michael S. Hershfield (Duke University School of Medicine) for providing a sample of *E. coli* transformed with a plasmid encoding for human placental AdoHcy hydrolase.

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