(*E*)-5′,6′-Didehydro-6′-deoxy-6′-fluorohomoadenosine: A Substrate That Measures the Hydrolytic Activity of *S*-Adenosylhomocysteine Hydrolase†

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ABSTRACT: (*E*)-5′,6′-Didehydro-6′-deoxy-6′-fluorohomoadenosine (EDDFHA), which is a poor substrate for the oxidative activity of *S*-adenosyl-L-homocysteine (AdoHcy) hydrolase and thus a poor mechanism-based inhibitor, was shown to be a good substrate for the hydrolytic activity of this enzyme. Incubation of EDDFHA with AdoHcy hydrolase (NAD^+ form) produces a large molar excess of hydrolytic products [e.g., fluoride ion, adenine (Ade) derived from chemical degradation of homoadenosine 6′-carboxaldehyde (HACA), and 6′-deoxy-6′-fluoro-5′-hydroxyhomoadenosine (DFHHA)] accompanied by a slow irreversible inactivation of the enzyme. The enzyme inactivation was shown to be time-dependent, biphasic, and concomitant with the reduction of the enzyme-bound NAD^+ (E·NAD^+) to E·NADH. The reaction of EDDFHA with AdoHcy hydrolase was shown to proceed by three pathways: pathway a, water attack at the 6′-position of EDDFHA and elimination of fluoride ion results in the formation of HACA, which degrades chemically to form Ade; pathway b, water attack at the 5′-position of EDDFHA results in the formation of DFHHA; and pathway c, oxidation of EDDFHA results in formation of the NADH form of the enzyme (inactive) and 3′-keto-EDDFHA, which could react with water at either the C5′ or C6′ positions. The partition ratios among the three pathways were determined to be $k_{3'}:k_{6'}:k_{5'}$ = 1:29:79 with one lethal event (enzyme inactivation) occurring every 108 nonlethal turnovers. Evidence in support of these mechanisms includes the observations that incubation of EDDFHA with AdoHcy hydrolase (NAD^+ form) generates a larger molar excess of fluoride ion [determined by ^{19}F nuclear magnetic resonance spectroscopy (NMR)], Ade [determined by high-performance liquid chromatography (HPLC) and chemical ionization mass spectrometry (CI-MS)], and DFHHA [determined by ^{19}F NMR, ^1H NMR, and fast atom bombardment mass spectrometry (FAB-MS)]. In an earlier study we have shown that the formation of Ade was a measure of HACA production [Yuan, C. S., Liu, S., Wnuk, S. F., Robins, M. J., & Borchardt, R. T. (1994) *Biochemistry 33*, 3758–3765]. DFHHA was shown in this study to be a weak reversible inhibitor of AdoHcy hydrolase. In addition, incubation of EDDFHA with the NADH form of the enzyme also generates fluoride ion, Ade, and DFHHA. The results from these studies have provided valuable new insights into the hydrolytic activity of AdoHcy hydrolase, and EDDFHA has been identified as a "fairly specific" substrate for measuring the hydrolytic activity independent of the oxidative activity.

S-Adenosyl-L-homocysteine (AdoHcy)[1] hydrolase (EC 3.3.1.1) is an eukaryotic enzyme that catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and homocysteine (Hcy) (de la Haba & Cantoni, 1959). The enzyme plays an important role in regulating biological methylation reactions by controlling the intracellular levels of AdoHcy (Kredich & Hershfield, 1979; Ueland, 1982), which is a potent product inhibitor of all *S*-adenosylmethionine (AdoMet)-dependent transmethylation reactions (Borchardt, 1980). Recently, AdoHcy hydrolase has attracted attention as a target for the design of antiviral agents (DeClercq, 1987; Keller & Borchardt, 1988; Wolfe & Borchardt, 1991; Liu *et al.*, 1992a,b, 1993) because inhibition of cellular AdoHcy hydrolase results in intracellular accumulation of AdoHcy and subsequent inhibition of all AdoMet-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).

Palmer and Abeles (1979) elucidated the mechanism by which AdoHcy hydrolase catalyzes the conversion of AdoHcy to Ado and Hcy. The first step (oxidative activity) involves oxidation of the 3′-hydroxyl group of AdoHcy by enzyme-bound NAD^+ (E·NAD^+) to form E·NADH and 3′-keto-AdoHcy, which undergoes β-elimination of Hcy to form 3′-keto-4′,5′-didehydro-5′-deoxy-Ado. Michael-type addition of water to this tightly bound intermediate (hydrolytic activity) affords 3′-keto-Ado, which is reduced by E·NADH to yield Ado and E·NAD^+. This mechanism implied that breakage of the C5′–S bond (elimination of Hcy from 3′-keto-AdoHcy) and formation of the C5′–O bond (addition of water to 3′-keto-4′,5′-didehydro-5′-deoxy-Ado) were dependent on the oxidative activity of the enzyme.

Yuan *et al.* (1993) have recently shown that AdoHcy hydrolase can catalyze addition of water to the 4′,5′ double

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[1] Abbreviations: EDDFHA, (*E*)-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine; EDDHHAs, (*E*)-5′,6′-didehydro-6′-deoxy-6′-halohomoadenosines; ZDDFA, (*Z*)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine; DFHHA, 6′-deoxy-6′-fluoro-5′-hydroxyhomoadenosine; HACA, homoadenosine 6′-carboxaldehyde; AdoHcy, adenosyl-L-homocysteine; E·NAD^+, enzyme-bound nicotinamide adenine dinucleotide; E·NADH, enzyme-bound nicotinamide adenine dinucleotide (reduced); BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; TMS, tetramethylsilane; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; CI-MS, chemical ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry.

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bond of (*Z*)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (ZDDFA) without its prior oxidation to the 3′-keto derivative. The resulting product, Ado-5′-carboxaldehyde, was shown to be a potent, mechanism-based inhibitor of AdoHcy hydrolase (Liu *et al.*, 1993). Ado-5′-carboxaldehyde, like many other inhibitors of this enzyme (Wolfe & Borchardt, 1991; Liu *et al.*, 1992a,b, 1993), serves as an alternative substrate for the oxidative activity of AdoHcy hydrolase, causing the conversion of E·NAD^+ (active) to E·NADH (inactive). This observation was of interest because of the potent inhibitory effects of ZDDFA and Ado-5′-carboxaldehyde on AdoHcy hydrolase and was also of mechanistic interest because it showed for the first time that the enzyme performed hydrolytic catalysis independent of the oxidation reaction. However, since the partition ratio (turnover events to inactivation events) of ZDDFA was close to 0, it was not practical to use this molecule as a substrate to probe the hydrolytic activity of the enzyme.

Recently, Wnuk *et al.* (1994) reported the synthesis of a series of homovinyl halide analogs [(*E*)-5′,6′-didehydro-6′-deoxy-6′-halohomoadenosines (EDDHHAs), halide = F, Cl, Br, I] of ZDDFA. The EDDHHAs, where the halide = Cl, Br, or I, have been shown to be substrates for the hydrolytic activity of AdoHcy hydrolase, resulting in addition of the hydroxyl group of water at C6′ with accompanying elimination of halide ion (Cl^-, Br^-, I^-) (Yuan *et al.*, 1994). The resulting homoadenosine-6′-carboxaldehyde (HACA) was chemically unstable and underwent degradation to adenine (Ade) and a homoribosyl fragment. Chemical degradation of HACA, which is formed at the active site of the enzyme, must be faster than its oxidation by AdoHcy hydrolase since, unlike ZDDFA, which had a partition ratio of 0, the partition ratios of the EDDHHAs (halide = Cl, Br, I) were shown to vary from 6 to 30 depending upon the halide. These results indicate that the EDDHHAs are relatively poor inhibitors of AdoHcy hydrolase but are partially selective for measuring the hydrolytic activity of the enzyme. However, in our previous study on EDDHHAs (halide = Cl, Br, or I), we were not able to isolate and characterize the direct reaction products generated enzymatically due to the chemical instability of the C6′ reaction product (HACA) and to the low partition of the C5′ reaction whose existence was not realized at that time.

In this paper, we describe the mechanism of interaction of the fluoro analog [(*E*)-5′,6′-didehydro-6′-deoxy-6′-fluorohomoadenosine (EDDFHA)], which undergoes water attack predominantly at the C5′ rather than the C6′ position as observed with EDDHHAs (halide = Cl, Br, or I). We have demonstrated that the reaction of EDDHHAs with AdoHcy hydrolase actually proceeds via three pathways instead of two as described previously, and the C5′ reaction product has been isolated and extensively characterized. In addition, EDDFHA shows a much large partition ratio (1 per 108 turnovers) than its Cl, Br, and I counterparts, which makes it an excellent substrate for measuring the hydrolytic activity of AdoHcy hydrolase independent of its oxidative activity.

MATERIALS AND METHODS

Materials. AdoHcy, Ado, Hcy, NAD^+, NADH, bakers' yeast alcohol dehydrogenase (EC 1.1.1.1), and beef heart lactate dehydrogenase (EC 1.1.1.27) were purchased from Sigma Chemical Co. (St. Louis, MO). EDDFHA was synthesized according to the described procedures (Wnuk *et al.*, 1994), and its structure is shown in Scheme 2.

Purification of AdoHcy Hydrolase (NAD⁺ Form). The recombinant human placental AdoHcy hydrolase was purified from the cell-free extract of *Escherichia coli* transformed with the plasmid pPROKcd20 and grown in the presence of isopropyl β-D-thiogalactopyranoside by a procedure reported earlier (Yuan *et al.*, 1993). The purification included DEAE-cellulose ion exchange (batch method) of the cell-free extract, $(NH_4)_2SO_4$ fractionation (30–60%), and Sephacryl S-300 and DEAE-Sepharose column chromatographies. About 50–60 mg of homogeneous enzyme (0.9–1.1 units/mg) was obtained from a 1-L culture. The subunit M_r (45 000) was used to calculate the molarity of the enzyme solutions. One unit of enzyme activity is defined as the amount of enzyme which can hydrolyze 1 μmol of AdoHcy to Ado and Hcy/min. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Preparation of the Apo and NADH Forms of AdoHcy Hydrolase. Apo-AdoHcy hydrolase was prepared by treatment of AdoHcy hydrolase (NAD^+ form) with $(NH_4)_2SO_4$ at pH 3.3 to remove the enzyme-bound NAD^+ as described earlier (Yuan *et al.*, 1993). The NADH form of AdoHcy hydrolase was obtained by reconstitution of the prepared apoenzyme with NADH (Yuan *et al.*, 1993).

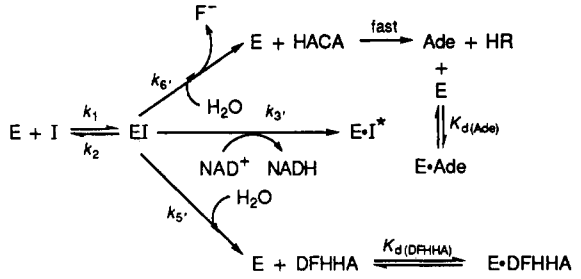
Assay of AdoHcy Hydrolase Activity. AdoHcy hydrolase activity was assayed in the synthetic direction by measuring the rate of formation of AdoHcy from Ado and Hcy. The enzyme was incubated with 0.2 mM Ado and 5 mM Hcy in 500 μL of 50 mM potassium phosphate buffer, pH 7.2, containing 1 mM EDTA (buffer A) at 37 °C for 5 min. The reaction was terminated by the addition of 25 μL of 5 N $HClO_4$. After the reaction mixture was kept in ice water for 15 min, the clear supernatant was collected and analyzed for AdoHcy by HPLC using a C-18 reversed-phase column (Econosphere C18, 5 μm, 250 × 4.6 mm, Alltech, Deerfield, IL) as described earlier (Yuan *et al.*, 1993).

Reaction of the Enzyme and EDDFHA. Time-dependent inactivation of AdoHcy hydrolase by EDDFHA was measured by incubating various concentrations of the inhibitor (2.5–15 μM) with 10 nM AdoHcy hydrolase in buffer A at 37 °C for different amounts of time (0–20 min). The activity remaining was determined in the synthetic direction as described above. Pseudo-first-order rate constants (k_{app}) were obtained from the initial kinetics of the reaction.

Determination of Fluoride Ion Released from EDDFHA. Fluoride ion released from the reaction of AdoHcy hydrolase and EDDFHA was determined by ^{19}F NMR spectroscopy recorded at 470.3 MHz on a Bruker AM spectrometer interfaced to an Aspect 300 computer. AdoHcy hydrolase (1.0 mg) was incubated with EDDFHA (0.80 mg) in 1 mL of buffer A at 37 °C for 1 h. To the reaction mixture was added 3 volumes of 95% alcohol to precipitate the protein. The supernatant from centrifugation was lyophilized. The residue was dissolved in 0.5 mL of D_2O for measurement of the ^{19}F NMR spectrum. NaF was used as an external reference.

Isolation and Characterization of the Products Produced by Incubation of EDDFHA with AdoHcy Hydrolase. AdoHcy hydrolase (3.0 mg) and EDDFHA (2.0 mg) were incubated in 1 mL of buffer A at 37 °C for 1 h. The reaction mixture was filtered through an Amicon Centricon-30 microconcentrator and the retentate on the membrane was washed with water (0.3 mL) once and centrifuged again. The filtrate and washing were combined and injected into an HPLC column (Econosphere, C18, 5 μm, 250 × 4.6 mm). The column was eluted at a flow rate of 1 mL/min in gradients of 6–25% B in A over 0–15 min and 25–40% B in A over 15–20 min, where mobile phase A was 50 mM sodium phosphate buffer, pH 3.2, containing 10 mM of 1-heptanesulfonic acid and mobile phase B was acetonitrile. The UV detector was set at 258 nm.

Scheme 1



Fractions from the main product peaks were collected and lyophilized.

Characterization of Ade formed was performed by HPLC and CI-MS method as reported previously (Yuan *et al.*, 1994). DFHHA was characterized by ^{19}F NMR, ^1H NMR, and fast atom bombardment-mass spectroscopy (FAB-MS). For the ^{19}F NMR, the experimental conditions were the same as described above for the fluoride ion assay. For ^1H NMR, the purified and lyophilized sample (510 μg) was dissolved in 50 μL of DMSO-d_6 and 400 μL of acetone-d_6 and the spectrum was obtained on a Varian XL-300 spectrometer. ^1H chemical shifts (δ, ppm) are relative to the internal standard tetramethylsilane (TMS, δ 0.00). FAB-MS was obtained on a mass spectrometer (ZABHS Model, VG Analytical Ltd., Manchester, U.K.) equipped with a 11/250 data system. A sample in water (20 μg/μL) was added to 3-nitrobenzyl alcohol as the matrix.

Products generated from the reaction of AdoHcy hydrolase (NADH form) and EDDFHA were characterized by HPLC comparison with the products generated from the reaction of AdoHcy hydrolase (NAD^+ form) and EDDFHA.

Determination of E·NAD⁺ and E·NADH. Quantitation of E·NAD^+ and E·NADH was performed using a fluorescence method reported previously (Yuan *et al.*, 1993). NADH released from the enzyme by ethanol precipitation was measured directly at an excitation wavelength of 340 nm and an emission wavelength of 460 nm, whereas NAD^+ was first converted to NADH by addition of bakers' yeast alcohol dehydrogenase.

EDDFHA-induced reduction of E·NAD^+ to E·NADH was determined by incubation of AdoHcy hydrolase (NAD^+ form) (100 μg) with 2 mM EDDFHA in buffer A (200 μL) at 37 °C for 1 h before addition of 3 volumes of ethanol. The supernatant from centrifugation was measured for both NAD^+ and NADH as described above.

Determination of Partition Ratios. Partition ratios for the enzyme inactivation induced by EDDFHA were determined kinetically by measuring the residual enzyme activity and the amount of the products formed, assuming the enzyme reaction mechanism shown in Scheme 1, where EI, E·Ade, and E·DFHHA are the reversible complexes of enzyme (NAD^+ form) with EDDFHA, Ade, and DFHHA, respectively, and E·I* is the irreversibly formed enzyme (NADH form)–3′-keto-EDDFHA complex. NAD^+ and NADH, which are tightly enzyme-bound coenzymes of AdoHcy hydrolase, do not dissociate from the enzyme in the reaction. HACA and DFHHA are products formed, and k_1, k_2, $k_{3'}$, $k_{5'}$, and $k_{6'}$ are the applicable rate constants. Ade and HR (homoribosyl moiety) are chemical degradation products from HACA. Both Ade and DFHHA are competitive inhibitors of the enzyme with K_i values of 85 μM and 1.1 mM, respectively, as determined from independent experiments. For the rate of the irreversible inhibition, the loss of active enzyme could be expressed as

$$-\frac{dE}{[E]} = \frac{k_{3'}[I]}{[I] + K_i\left(1 + \frac{[Ade]}{K_{d(Ade)}} + \frac{[DFHHA]}{K_{d(DFHHA)}}\right)}dt \approx \frac{k_{3'}[I]}{[I] + K_i\left(1 + \frac{[Ade]}{K_{i(Ade)}} + \frac{[DFHHA]}{K_{i(DFHHA)}}\right)}dt \quad (1)$$

Since the concentrations of reaction products [Ade] and [DFHHA] are very low in the initial reaction compared with the "substrate" [EDDFHA], and their K_i values are relatively large, it is assumed that the initial kinetics is approximately equal to

$$-\frac{dE}{[E]} = \frac{k_{3'}[I]}{[I] + K_i}dt \quad (2)$$

Integration of (2) gives

$$\ln\frac{[E]}{[E_0]} = \frac{k_{3'}[I]}{[I] + K_i}t \quad (3)$$

If $[I] \gg [E_0]$, let $k_{3'}[I]/([I] + K_i) = k_{app}$ and then

$$\frac{1}{k_{app}} = \frac{1}{k_{3'}} + \frac{K_i}{k_{3'}[I]} \quad (4)$$

A double-reciprocal plot of $1/k_{app}$ *versus* $1/[I]$ gives a slope of $K_i/k_{3'}$ and a y intercept of $1/k_{3'}$.

The formation rate of the product HACA can be expressed as

$$\frac{d(HACA)}{dt} = k_{6'}[EI] \quad (5)$$

$$\frac{d(HACA)}{dt}\frac{1}{[E]} = \frac{k_{6'}[I]}{[I] + K_i} = k_{app}\frac{k_{6'}}{k_{3'}} \quad (6)$$

as

$$[E] = [E_0]e^{-k_{app}t} \quad (7)$$

so

$$\frac{d(HACA)}{dt} = k_{app}\frac{k_{6'}}{k_{3'}}[E_0]e^{-k_{app}t} \quad (8)$$

Integration of (8) gives

$$(HACA)_t = \frac{k_{6'}}{k_{3'}}([E_0] - [E_t]) = \frac{k_{6'}}{k_{3'}}[E_0](\%\ \text{inhibition})/100 \text{ at time } t \quad (9)$$

In the same way, the formation rate of the product DFHHA can be expressed as

$$(DFHHA)_t = \frac{k_{5'}}{k_{3'}}([E_0] - [E_t]) = \frac{k_{5'}}{k_{3'}}[E_0](\%\ \text{inhibition})/100 \text{ at time } t \quad (10)$$

where k_{app} is the pseudo-first-order rate constant, K_i is defined as $(k_2 + k_{3'} + k_{5'} + k_{6'})/k_1$, and $[E_0]$ and $[E_t]$ ($[E_t] = [E_0]e^{-k_{app}t}$)

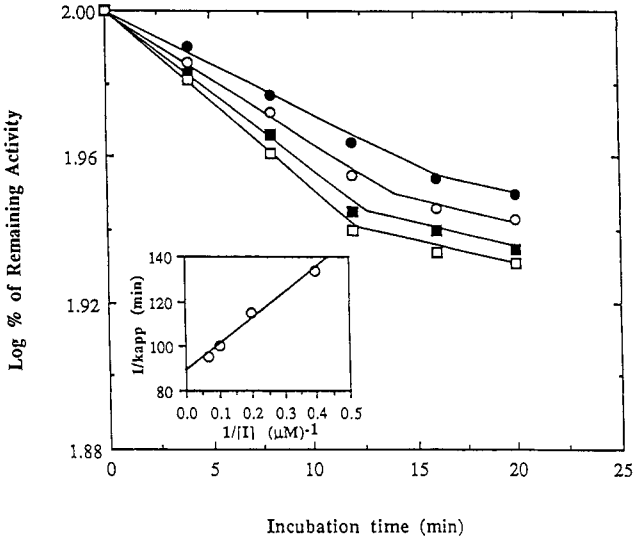


FIGURE 1: Time-dependent inactivation of AdoHcy hydrolase by EDDFHA. AdoHcy hydrolase (10 nM) was incubated with EDDFHA (●, 2.5 μM; ○, 5 μM; ■, 10 μM; □, 15 μM) in buffer A at 37 °C for various times. At the indicated time points, residual enzyme activity was determined in the synthetic direction using Ado and Hcy as substrates as described under Materials and Methods. k_{app} values were calculated at each concentration of EDDFHA from the slopes of the linear part of the plot. (Inset) Plot of $(k_{app})^{-1}$ *versus* $[I]^{-1}$ from which the K_i and $k_{3'}$ values were calculated as described under Materials and Methods. Data were the average of duplicate measurements.

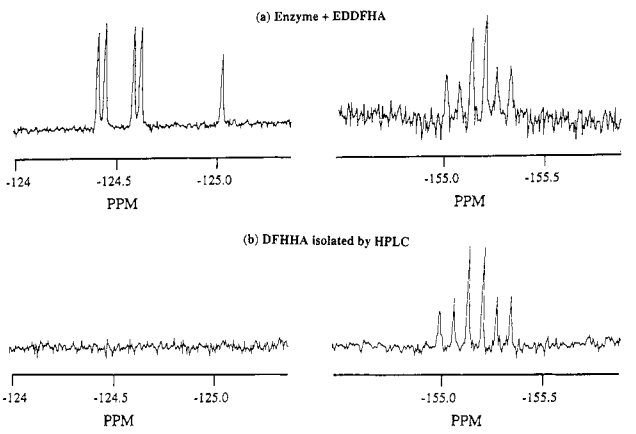


FIGURE 2: ^{19}F NMR spectra of EDDFHA incubated with AdoHcy hydrolase. (a) AdoHcy hydrolase (1.0 mg) was incubated with EDDFHA (800 μg) in 1 mL of buffer A at 37 °C for 1 h. The reaction mixture was then treated with 95% of ethanol, centrifuged, and lyophilized as described under Materials and Methods. ^{19}F NMR spectra were recorded in D_2O with a Bruker AM 500 spectrometer. (b) Sample (peak B) isolated from the reaction mixture of AdoHcy hydrolase and EDDFHA by HPLC as described under Materials and Methods.

are the active enzyme concentrations at initial and at reaction time t, respectively.

In this experiment, AdoHcy hydrolase (5 μM) was incubated with 800 μM EDDFHA in buffer A at 37 °C for different times (2–10 min). At each time point, two samples (5 and 100 μL) were withdrawn from the reaction mixture, one to determine the percent activity remaining and one to quantify the Ade (formed from chemical degradation of HACA) and DFHHA, respectively. For the assay of percent enzyme activity remaining, the reaction mixture was added to a test tube containing 545 μL of buffer A and frozen immediately in an acetone–dry ice bath. After thawing at 0 °C, 10 μL of 10 mM Ado and 40 μL of 62.5 mM Hcy were added to the reaction mixture and incubation was conducted at 37 °C for 5 min. The reaction was stopped by adding 20 μL of 5 N $HClO_4$. The AdoHcy formed was determined by HPLC as described above. For the measurements of Ade and DFHHA, the reaction mixture was added to a test tube containing 5 μL of 5 N $HClO_4$. The supernatant from centrifugation and the washing (100 μL of H_2O) were combined and subjected to HPLC analysis as described above.

RESULTS

Inactivation of AdoHcy Hydrolase by EDDFHA. Inactivation of purified AdoHcy hydrolase with a large molar excess of EDDFHA resulted in time-dependent loss of enzyme activity, as shown in Figure 1. The rate of the inactivation appeared to be biphasic, showing pseudo-first-order kinetics in the initial inactivation period (in about 10 min) followed by a slower inactivation rate when about 15% of the enzyme was inactivated. A double-reciprocal plot of the initial pseudo-first-order inactivation rate constants ($1/k_{app}$) *versus* EDDFHA concentrations ($1/[I]$) (Figure 1, inset) gave K_i and $k_{3'}$ values of 1.3 μM and 1.1×10^{-2} min^{-1}, respectively, according to eq 4. Inactivation of AdoHcy hydrolase by EDDFHA was irreversible since the enzyme activity lost could not be regained by prolonged dialysis as seen in Table 1.

Effect of EDDFHA on E·NAD⁺. Incubation of AdoHcy hydrolase with EDDFHA resulted in the reduction of E·NAD^+ to E·NADH, which was quantitatively determined by a fluorescence method. When about 50% of the enzyme was inactivated after 1 h of incubation with 2 mM EDDFHA, it was found that about the same percentage of E·NAD^+ was converted to E·NADH (Table 1).

Release of Fluoride Ion from EDDFHA upon Incubation with AdoHcy Hydrolase. Release of fluoride ion from EDDFHA upon incubation with AdoHcy hydrolase (NAD^+ form) was determined by ^{19}F NMR spectroscopy. The ^{19}F NMR spectrum of EDDFHA had a doublet of doublets at −124.5 ppm (J_1 = 17.0 Hz, J_2 = 83.5 Hz). When a large molar excess of EDDFHA was incubated with AdoHcy hydrolase, a singlet fluoride ion signal at −125.0 ppm and a multiplet signal for a fluorine-containing compound at −155.2 ppm (J_1 = 25.4 Hz, J_2 = 46.6 Hz) were observed in addition to the signal from the unreacted EDDFHA (−124.5 ppm) (Figure 2a). Incubation of either enzyme or EDDFHA alone did not result in the appearance of the singlet and multiplet in the ^{19}F NMR spectrum (data not shown).

Isolation and Characterization of the Products Generated from Reaction of EDDFHA and AdoHcy Hydrolase. After incubation of EDDFHA with AdoHcy hydrolase and removal of the enzyme by filtration through an Amicon Centricon-30 membrane, the filtrate was injected into an HPLC system to separate the generated reaction products. As shown in Figure

Table 1: Effect of EDDFHA on the Enzyme-Bound NAD^+ and NADH Contents of AdoHcy Hydrolase[a]

sample	NAD^+ content (mol/mol of enzyme subunit)	NADH content (mol/mol of enzyme subunit)	% activity remaining	
			before dialysis	after dialysis
enzyme	0.72 ± 0.06	0.21 ± 0.06	100	100
enzyme + EDDFHA	0.44 ± 0.04	0.53 ● 0.04	54.8 ± 1.2	53.4 ± 1.8

[a] NAD^+ and NADH contents were determined by a fluorescence method as described in Materials and Methods.

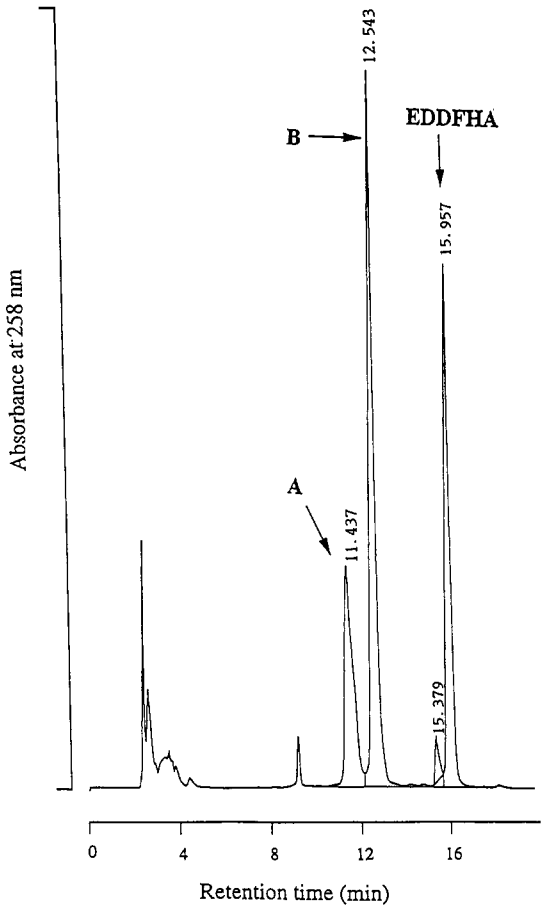


FIGURE 3: HPLC chromatogram of the products generated upon incubation of AdoHcy hydrolase (NAD^+ form) and EDDFHA. AdoHcy hydrolase (3.0 mg) was incubated with EDDFHA (2.0 mg) in 1 mL of buffer A at 37 °C for 1 h. The reaction mixture was filtered through an Amicon Centricon-30 microconcentrator. An aliquot of the filtrate (10 μL) was injected into an HPLC system for analysis as described under Materials and Methods. Absorbance was monitored at 258 nm.

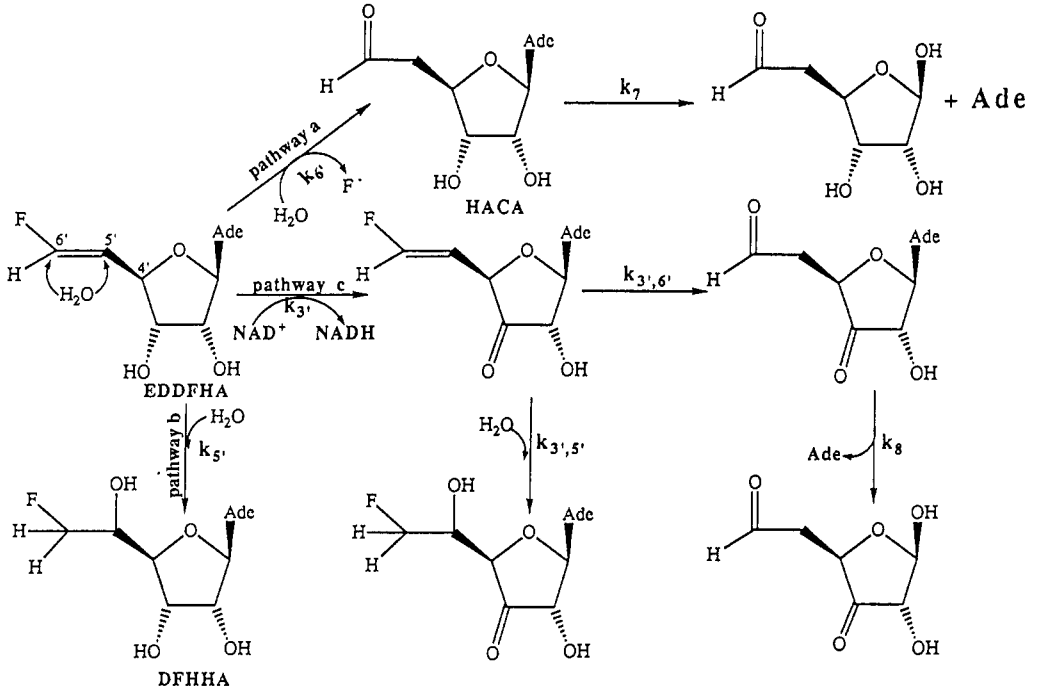
3, two new peaks with retention times of 11.4 min (peak A) and 12.5 min (peak B) were observed. The unreacted EDDFHA was eluted at a retention time of 16.0 min. The ratio of peak B to peak A was determined to be 2.65. The sample collected from peak A was identified as Ade by coinjection on HPLC with an authentic Ade sample and by CI-MS analysis which showed an ion at m/z 136 (MH^+). The sample collected from peak B was subjected to ^{19}F NMR analysis. As shown in Figure 2b, a multiplet at −155.2 ppm was observed which was identical to the multiplet observed in the reaction mixture of EDDFHA and AdoHcy hydrolase (Figure 2a). Further characterization of peak B was carried out by ^1H NMR and FAB-MS analyses, which gave the following results: ^1H NMR (acetone-d_6 + DMSO-d_6) δ 8.26 (s, 1 H, H-8), 8.19 (s, 1 H, H-2), 5.93 (d, J = 7.11 Hz, 1 H, H-1′), 4.88 (dd, J_1 = 5.04 Hz, J_2 = 7.05 Hz, 1 H, H-2′), 4.52 (dm, J = 48 Hz, 2 H, H-6′), 4.41 (m, 1 H, H-3′), 4.18 (m, 1 H, H-4′), 4.12 (dm, J = 23 Hz, 1 H, H-5′); FAB-MS m/z = 300. The ^{19}F NMR, ^1H NMR, and FAB-MS data are all consistent with the structure of DFHHA (see DFHHA in Scheme 2).

Ade is a competitive inhibitor of human AdoHcy hydrolase with a K_i value of 85 μM. The reaction product DFHHA was found to be a very weak competitive inhibitor of the enzyme (K_i = 1.1 mM), and the inhibition was not time-dependent.

Incubation of EDDFHA with the NADH form of AdoHcy hydrolase also resulted in the formation of fluoride ion, Ade, and DFHHA (data not shown). However, these hydrolytic products were not observed when EDDFHA was incubated with apo-AdoHcy hydrolase.

Partition Ratios. The partition ratio or number of turnovers per inactivation event is an important indicator of potency for irreversible mechanism-based inhibitors. After presumed reversible binding of EDDFHA to AdoHcy hydrolase, three apparent pathways were observed, which involve the enzyme-catalyzed formation of (a) fluoride ion and Ade, (b) DFHHA, and (c) 3′-keto derivatives of EDDFHA as measured by the reduction of E·NAD^+ to E·NADH (inactivation event). Partition ratios for these three pathways were determined kinetically by plotting the amount of the enzyme inactivated *versus* the amount of product (Ade or DFHHA) formed (eqs 9 and 10). The slopes from these plots give the values of

Scheme 2: Mechanisms for Conversions of EDDFHA to Products by AdoHcy Hydrolase



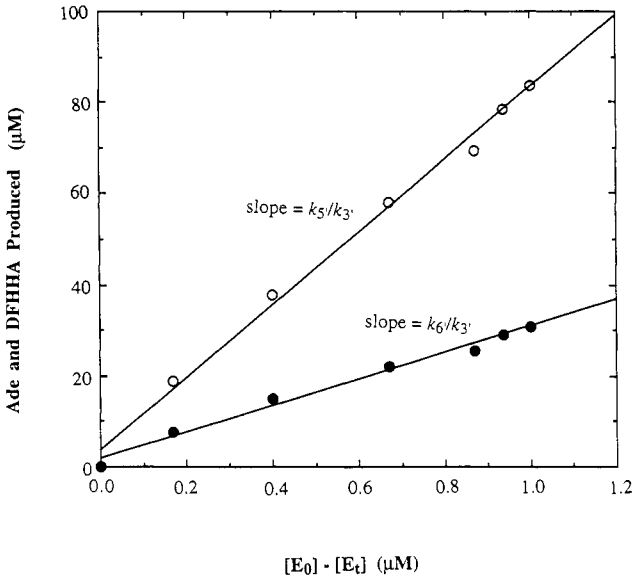


FIGURE 4: Relationship between turnovers and inactivation of AdoHcy hydrolase by EDDFHA. AdoHcy hydrolase (5 μM) was incubated with 800 μM EDDFHA in buffer A at 37 °C for various times. At each time point as indicated, residual enzyme activity and Ade and DFHHA formed were determined as described under Materials and Methods. $[E_0] - [E_t]$ (equal to $[E_0]$(% inactivtion)/100) was plotted *versus* the concentration of Ade or DFHHA formed, and $k_{6'}/k_{3'}$ or $k_{5'}/k_{3'}$ were calculated from the slopes according to eqs 9 and 10 as described under Materials and Methods. Data were the average of duplicate measurements (●, Ade; ○, DFHHA).

$k_{6'}/k_{3'}$ or $k_{5'}/k_{3'}$ (Figure 4). Since $k_{3'}$ was solved from eq 8, both $k_{6'}$ and $k_{5'}$ could be obtained from the plots, and the values of the partition ratios could be determined. The values of $k_{6'}$ and $k_{5'}$ were found to be 0.312 min^{-1} and 0.858 min^{-1}, respectively. Thus, the partition ratios of $k_{6'}/k_{3'}$, $k_{5'}/k_{3'}$, $k_{5'}/k_{6'}$, and $(k_{6'} + k_{5'})/k_{3'}$ were 29, 79, 2.74, and 108, respectively.

DISCUSSION

Inactivation of AdoHcy hydrolase by EDDFHA was observed to be time- and concentration-dependent, and the rate of inactivation appeared to be biphasic. Since both Ade and DFHHA are reversible inhibitors of AdoHcy hydrolase, competition between EDDFHA and its hydrolytic products (Ade and DFHHA) for binding with the enzyme becomes significant when Ade and DFHHA accumulate later in the inactivation process. Thus, the enzyme is competitively protected from irreversible inhibition by the progressively increasing reversible binding of the products Ade and DFHHA. This protection is evident in the biphasic inactivation time profiles (Figure 1). Similar biphasic kinetics were observed when alanine racemase was inactivated by mechanism-based inhibitors such as 3-halovinylglycines (Thornberry *et al.*, 1991). However, since the concentrations of the hydrolytic products (Ade and DFHHA) were still lower than their K_i values, other factors which contribute to the biphasic kinetics also have to be considered.

Incubation of AdoHcy hydrolase with EDDFHA resulted in the formation of a large molar excess of fluoride ion, Ade, and DFHHA, and reduction of E·NAD^+ to E·NADH. This indicates that at least three different catalytic pathways are involved in the process of enzyme inactivation by EDDFHA. Scheme 2 illustrates these proposed mechanisms. In pathway a, which was demonstrated earlier (Yuan *et al.*, 1994), the first step is addition of the hydroxyl group of water to the C6′ of EDDFHA and the expulsion of fluoride ion, resulting in the formation of HACA. HACA is chemically unstable and spontaneously degrades to Ade and a homoribosyl derivative by a retro-Michael addition mechanism (Yuan *et al.*, 1994). In pathway b, water attack of the hydroxyl group at C5′ of EDDFHA results in the formation of DFHHA. The chemically stable DFHHA is released from the enzyme since it is a weak competitive inhibitor of AdoHcy hydrolase with a K_i value about 850 times greater than that of EDDFHA. Pathway c is a lethal inactivation pathway in which the first step is oxidation of EDDFHA to 3′-keto-EDDFHA by reduction of E·NAD^+ to E·NADH. The 3′-keto-EDDFHA might be tightly bound to the enzyme since it was not detected in the filtrate of the reaction mixture of AdoHcy hydrolase and EDDFHA. However, it might undergo reaction with water by hydroxyl attack at either C6′ ($k_{3',6'}$) to form Ade and the 3′-keto-sugar-6′-carboxaldehyde product or at C5′ ($k_{3',5'}$) to form 3′-keto-DFHHA (Scheme 2). Such 3′-keto nucleosides could undergo spontaneous chemical degradation upon further manipulation (Hansske *et al.*, 1984).

In our earlier study on the inactivation of AdoHcy hydrolase by EDDHHAs (Yuan *et al.*, 1994), it was demonstrated that incubation of these analogs with AdoHcy hydrolase resulted in the formation of large molar excesses of Ade from spontaneous degradation of HACA. However, another product was detected in the same reaction mixture which also eluted after the Ade peak on the HPLC chromatogram, and this peak was much smaller than that of Ade (Yuan *et al.*, 1994). In light of the present study, the unknown peak is likely to be 6′-deoxy-6′-halo-5′-hydroxyhomoadenosine formed by enzyme-catalyzed addition of the hydroxyl group of water at C5′ of the EDDHHAs. On the basis of these observations, it is concluded that AdoHcy hydrolase can catalyze addition of the hydroxyl group of water at both the C6′ and C5′ positions of EDDHHAs. The ratios of $k_{5'}/k_{6'}$ strongly depend on the properties of the halogen at C6′ and were shown to be in the order of F > Cl > Br > I (*i.e.*, 2.75:0.37:0.11:0.08). Similarly, the partition ratios (the ratios of turnover events to inactivation events) were also in the order of F > Cl > Br > I (*i.e.*, 108:38.9:12.8:7.0). The large partition ratio (108) for EDDFHA makes it a "fairly specific" substrate for unique measurement of the hydrolytic activity of the enzyme. The significantly greater electronegativity of fluorine relative to that of the other halogens has been invoked to rationalize the enhancement of the acidity of hydrogens on carbon atoms β to fluorine. In contrast, the other halogens enhance the acidity of geminal hydrogens on α carbons (Hudlicky, 1991). Parallel effects with our EDDHHAs would result in strong polarization of the 6′-fluorovinyl (EDDFHA) compound and thus promote rapid addition of the hydroxyl of water at C5′ to give the weakly bound DFHHA as observed. The geminal effect of the other three halogens would favor water addition by hydroxyl attack at C6′ followed by loss of a proton and halide to give the 6′-homoaldehyde HACA, which subsequently undergoes spontaneous retro-Michael decomposition with expulsion of Ade. The slower the rate of addition of water (C5′/C6′) to an EDDHHA in the active site, the greater the opportunity for oxidation at C3′ to occur. Interestingly, the observed inactivation of AdoHcy hydrolase by the EDDHHAs is in the order I > Br > Cl > F (Wnuk *et al.*, 1994).

Although the primary sequences of both rat liver and human placental AdoHcy hydrolase have been known for several years (Ogawa *et al.*, 1987; Coulter-Karis & Hershfield, 1989), little is known about the tertiary structures and key residues at the active site. Crystallography of AdoHcy hydrolase has been elusive because of difficulties in obtaining high-quality crystals necessary for X-ray diffraction. Alternative methods such as chemical modification (Gomi *et al.*, 1986), photoaffinity labeling (Aiyar & Hershfield, 1985), site-directed mutagenesis

(Gomi *et al.*, 1989; Ault-Riché *et al.*, 1994), and computer-aided molecular modeling (Yeh *et al.*, 1991) have been used in efforts to identify key amino acid residues involved in substrate binding and/or catalysis. The present discovery that AdoHcy hydrolase has hydrolytic activity independent of C3′ oxidative activity with EDDFHA has made this compound very useful in characterizing chemically modified AdoHcy hydrolases or site-directed mutants of the enzyme. Loss of enzyme activity with chemically modified enzymes or site-directed mutants could result from steric hindrance caused by insertion of chemical reagent(s) or conformational changes caused by mutations. Therefore, the availability of EDDFHA, which binds to the active site of AdoHcy hydrolase but primarily exhibits only one of the two major catalytic activities, will be helpful in dissecting more specific causes of losses of enzyme activity. For instance, a chemically modified AdoHcy hydrolase or site-directed mutant of the enzyme which has lost total catalytic activity might retain C5′ and/or C6′ hydrolytic activity with EDDFHA. This would indicate that modification or mutation had not prevented substrate binding, but the modified or mutated residue(s) might be involved with C3′ oxidation. Conversely, if the modified or mutated AdoHcy hydrolase [*e.g.*, mutant K426R (Ault-Riché *et al.*, 1994)] maintained its C3′ oxidative activity but lost its C5′ hydrolytic activity, the modified or mutated residue(s) might be involved in catalysis of the addition of water at C5′. In fact, EDDFHA could be used to distinguish among all three possibilities, *i.e.*, loss of binding ability, loss of C3′ oxidative activity, or loss of C5′/C6′ hydrolytic activity.

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