Adenine Nucleoside Dialdehydes: Potent Inhibitors of Bovine Liver *S*-Adenosylhomocysteine Hydrolase†

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ABSTRACT: Various ribonucleoside 2′,3′-dialdehydes, including adenosine dialdehyde, *S*-adenosylhomocysteine (AdoHcy) dialdehyde, and 5-(methylthio)-5′-deoxyadenosine (MTA) dialdehyde, were shown to be potent inhibitors of bovine liver AdoHcy hydrolase (EC 3.3.1.1). These ribonucleoside 2′,3′-dialdehydes produce both time-dependent and concentration-dependent inactivation of the AdoHcy hydrolase. The inactivation appears to be irreversible since the enzyme activity cannot be recovered after prolonged dialysis against phosphate buffer. However, a substantial percentage of the enzyme activity could be recovered when the inactivated enzyme was dialyzed against a nitrogen buffer [e.g., tris(hydroxymethyl)aminomethane (Tris)]. This reversal of inhibition could be prevented, however, by pretreatment of the ligand–enzyme complex with sodium borohydride prior to dialysis in Tris buffer. Inclusion of substrates (e.g., adenosine or AdoHcy) afforded protection of the enzyme from the inactivation induced by the ribonucleoside 2′,3′-dialdehydes. These data suggest that the bond formed between the enzyme and the inhibitor is probably a Schiff base linkage between the aldehydic functionality of the inhibitor and a protein lysinyl residue in or around the adenosine–AdoHcy binding site. When [2,8-^{3}H]adenosine dialdehyde was used, a stoichiometry of 1.73 nmol of inhibitor bound per nmol of AdoHcy hydrolase was determined. Analysis of the kinetics of enzyme inactivation using the Ackermann–Potter approach indicates that adenosine dialdehyde is a tight-binding inhibitor, exhibiting a stoichiometry of one to two molecules of inhibitor bound to one molecule (tetramer) of enzyme and a K_i = 2.39 nM.

S-Adenosyl-L-methionine (AdoMet)-dependent methyltransferases have been shown to play important roles in the biosynthesis and/or degradation of small molecules, e.g., dopamine, norepinephrine, epinephrine, histamine, and serotonin (Usdin et al., 1982; Borchardt, 1980a), and in the modulation of the activity of macromolecules, e.g., proteins, nucleic acids, and phospholipids (Usdin et al., 1982; Borchardt, 1980b). A general feature of most AdoMet-dependent methyltransferases is the product inhibition produced by *S*-adenosyl-L-homocysteine (AdoHcy). The inhibitory effects of AdoHcy on AdoMet-dependent transmethylations constitute part of a biological regulatory mechanism, with the other component being AdoHcy hydrolase (EC 3.3.1.1), the enzyme that metabolizes AdoHcy in eukaryotic systems (Cantoni et al., 1979).

AdoHcy hydrolase catalyzes both the forward (hydrolysis) and reverse (synthesis) reactions shown in eq 1. The K_{eq} for

$$\text{AdoHcy} + H_2O \rightleftharpoons \text{adenosine} + \text{L-homocysteine} \quad (1)$$

this reaction favors synthesis of AdoHcy; however, in vivo the reaction is pulled in the hydrolytic direction by the enzymatic removal of both adenosine and homocysteine (De la Haba & Cantoni, 1959). Palmer & Abeles (1979) have shown that the catalytic mechanism of AdoHcy hydrolase involves the NAD^+-dependent oxidation of the 3′-hydroxyl group of AdoHcy (hydrolytic direction) or adenosine (synthetic direction), resulting in the generation of 3′-ketoAdoHcy, 3′-ketoadenosine, and 3′-keto-4′,5′-dehydroadenosine as enzymatically bound intermediates (Figure 1).

Ribonucleoside 2′,3′-dialdehydes (Figure 1), which can be prepared by periodic oxidation of the corresponding ribonucleoside, resemble structurally the 3′-ketoribonucleoside intermediates generated in the AdoHcy hydrolase catalyzed reaction. Adenosine dialdehyde, an example of a ribonucleoside 2′,3′-dialdehyde, was found to be a potent AdoHcy hydrolase inhibitor (K_i = 3.3 nM) in vitro (Hoffman, 1979) and in vivo in mouse liver (Hoffman, 1980). In an earlier communication from our laboratory (Borchardt et al., 1982), we reported preliminary evidence that adenosine dialdehyde produces irreversible inhibition of purified bovine liver AdoHcy hydrolase and that the compound inhibits AdoHcy hydrolase in clone 929 mouse L cells. The inhibition of AdoHcy hydrolase in mouse L cells by adenosine dialdehyde results in increases in cellular levels of AdoHcy and subsequent inhibition of cellular AdoMet-dependent methyltransferases (Bartel & Borchardt, 1984). Adenosine dialdehyde is also an effective inhibitor of vaccinia virus multiplication in clone 929 mouse L cells (Keller & Borchardt, 1983).

In the present study, we report that AdoHcy dialdehyde and 5′-(methylthio)-5′-deoxyadenosine (MTA) dialdehyde are also irreversible inhibitors of AdoHcy hydrolase, exhibiting enzyme inactivation kinetics similar to those observed for adenosine dialdehyde. We further report that adenosine dialdehyde behaves as a pseudoirreversible or a tight-binding inhibitor of AdoHcy hydrolase, where one to two molecules of the inhibitor bind per tetramer of enzyme.

Materials and Methods

Source of Chemicals. Calf intestinal adenosine deaminase, dithiothreitol (DTT), aminohexylagarose, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Bio-Gel P-6 was obtained from Bio-Rad Laboratories (Richmond, CA).

[2,8-^{3}H]AdoHcy (19 μCi/μmol) was prepared by conversion of [2,8-^{3}H]adenosine (32 μCi/μmol; ICN, Irvine, CA) to 5′-chloro-5′-[2,8-^{3}H]deoxyadenosine followed by its condensation with L-homocysteine in sodium and liquid ammonia

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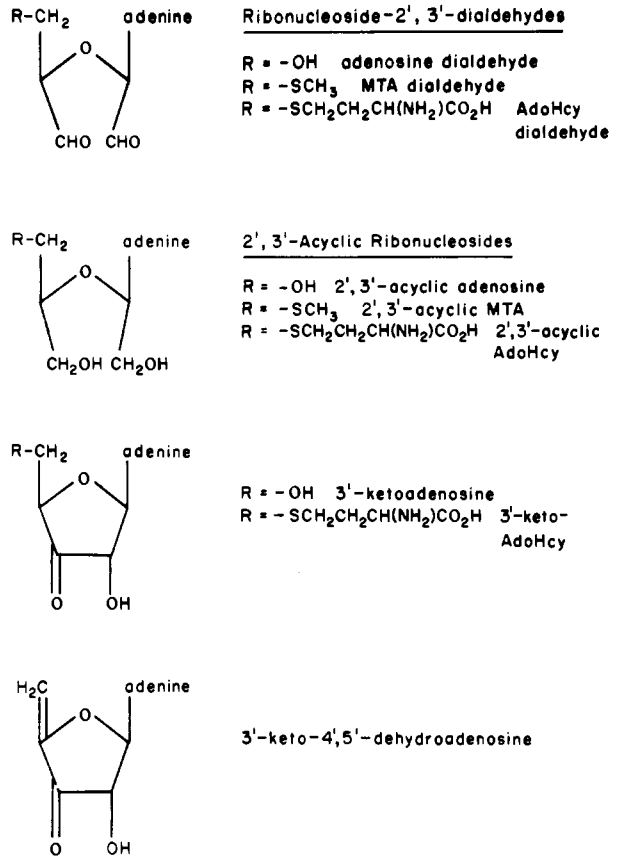

FIGURE 1: Comparison of structures of 3′-ketoadenosine, 3′-keto-AdoHcy, and 3′-keto-4′,5′-dehydroadenosine with ribonucleoside 2′,3′-dialdehydes and 2′,3′-acyclic ribonucleosides which are potential inhibitors of AdoHcy hydrolase.

(Borchardt et al., 1976). [2,5′,8-^{3}H]- and [2,8-^{3}H]adenosine dialdehyde (23 μCi/μmol), adenosine dialdehyde, and AdoHcy dialdehyde were prepared by paraperiodic acid oxidation (Borchardt et al., 1978) of [2,5′,8-^{3}H]- or [2,8-^{3}H]adenosine (Amersham, Arlington Heights, IL), adenosine (Sigma Chemical Co., St. Louis, MO), and AdoHcy, respectively. The chromatographic and spectral properties of these ribonucleoside 2′,3′-dialdehydes were identical with those reported earlier by Borchardt et al. (1978). MTA dialdehyde, which was prepared by paraperiodic acid oxidation of MTA (Borchardt, 1979), exhibited properties consistent with those reported by Moorman (1982).

2′,3′-Acyclic adenosine, 2′,3′-acyclic MTA, and 2′,3′-acyclic AdoHcy (Figure 1) were prepared by sodium borohydride reduction of the corresponding nucleoside dialdehydes (Borchardt et al., 1978; Moorman, 1982). The chromatographic and spectral properties of the 2′,3′-acyclic nucleosides were consistent with those reported earlier by Moorman (1982).

Purification of Calf Liver AdoHcy Hydrolase. To determine the general effects of ribonucleoside dialdehydes on AdoHcy hydrolase activity, a partially purified preparation of calf liver AdoHcy hydrolase was employed. The enzyme was isolated by using a modification of the procedure of Richards et al. (1978). The specific activities of these partially purified enzymes ranged from 8 to 60 nmol of inosine formed min^{-1} (mg of protein)$^{-1}$, representing a 10–35-fold purified enzyme.

To determine the stoichiometry of [2,8-^{3}H]adenosine dialdehyde incorporation and to determine the kinetics of enzyme inactivation, AdoHcy hydrolase was purified to homogeneity according to the procedure of Palmer & Abeles (1979). This procedure afforded a 663-fold purified preparation of AdoHcy hydrolase having a specific activity of 0.12 IU [1 IU is defined as 1 μmol of product formed min^{-1} (mg of protein)$^{-1}$]. This enzyme preparation was >95% pure as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Weber & Osborn, 1969). The enzyme used for Ackermann–Potter analysis was also purified according to the procedure of Palmer & Abeles (1979) with a final specific activity of 0.32 IU. The protein concentration (0.21 mg/mL) was determined by the absorbance at 280 nm, $E^{1\%}_{1cm}$ = 14.2 (Abeles et al., 1982), and by the procedure of Lowry et al. (1951). This enzyme preparation was >95% pure as judged by sodium dodecyl sulfate gel electrophoresis (Weber & Osborn, 1969).

Assay of AdoHcy Hydrolase in the Hydrolytic Direction. The assay of AdoHcy hydrolase activity in the hydrolytic direction was determined by a modification of the procedure of Richards et al. (1978). In a total volume of 500 μL, the incubation mixture contained 150 mM potassium phosphate buffer (pH 7.6), 1.0 mM EDTA, 100 μM [2,8-^{3}H]AdoHcy, and 4 units of intestinal adenosine deaminase. The reaction was started by the addition of AdoHcy hydrolase and incubated 5 min at 37 °C. The reaction was terminated by the addition of 100 μL of 5 N formic acid. The reaction mixture and a 500-μL wash of 0.1 N formic acid were layered onto a column of SP-Sephadex C-25 (1.2 × 2.4 cm), previously equilibrated in 0.1 N formic acid. The column was eluted with 8.0 mL of 0.1 N formic acid, and the eluent containing [2,8-^{3}H]inosine (the product of the hydrolysis of [2,8-^{3}H]-AdoHcy and subsequent deamination of [2,8-^{3}H]adenosine) was collected. A 1-mL aliquot was added to 10 mL of 3a70 scintillation cocktail (Research Products International, Mount Prospect, IL) and the amount of radioactivity determined by liquid scintillation spectrometry.

Reversibility of AdoHcy Hydrolase Inactivated by Ribonucleoside 2′,3′-Dialdehydes. The standard preincubation mixture consisting of 150 mM potassium phosphate buffer (pH 7.6), EDTA (1.0 mM), the indicated amount of inhibitor, and AdoHcy hydrolase was incubated at 28 °C for 5 min (Table I). Samples in duplicate were assayed for enzyme activity at time zero and at the end of the preincubation time and after dialysis. After preincubation, samples were either dialyzed directly against 150 mM potassium phosphate buffer (pH 7.6) containing DTT (5 mM) and EDTA (1 mM) for 16 h or treated with an equal volume of 1.0 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 7.6) containing DTT (5 mM) and EDTA (1.0 mM) at 28 °C for 4.5 h prior to dialysis against the same Tris buffer for 16 h at 4 °C. In some experiments, the preincubation mixtures were first treated with potassium borohydride (5× concentration of inhibitor) at 28 °C for 2 h and then with an equal volume of 1.0 M Tris buffer (pH 7.6) containing DTT (5 mM) and EDTA (1.0 mM) for 4.5 h, after which the samples were dialyzed against the same Tris buffer for 16 h. The residual enzyme activity was calculated relative to the activity of AdoHcy hydrolase treated in an identical manner except for the omission of the ribonucleoside 2′,3′-dialdehydes.

RESULTS

General Characteristics of AdoHcy Hydrolase Inactivation Induced by Ribonucleoside 2′,3′-Dialdehydes. In a preliminary communication (Borchardt et al., 1982), we reported that adenosine dialdehyde is a potent irreversible inhibitor of AdoHcy hydrolase. In this study, we have found that AdoHcy dialdehyde and MTA dialdehyde are also potent inhibitors of AdoHcy hydrolase (Table I shows data for AdoHcy dialdehyde compared to adenosine dialdehyde), whereas the corresponding 2′,3′-acyclic ribonucleosides are substantially less effective as

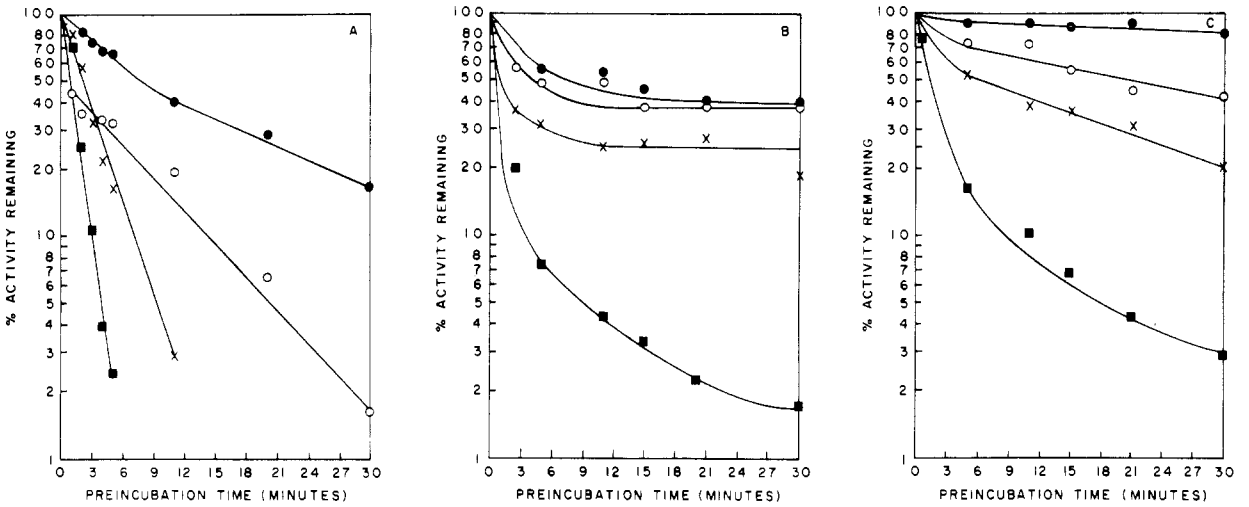

FIGURE 2: Effect of ribonucleoside 2′,3′-dialdehydes on the rate of inactivation of AdoHcy hydrolase. The preincubation mixtures consisted of the following inhibitors: AdoHcy dialdehyde (A) [(●) 0.5 μM; (○) 1 μM; (×) 2 μM; (■) 5 μM], adenosine dialdehyde (B) [(●) 200 nM; (○) 225 nm; (×) 235 nM; (■) 240 nM], or MTA dialdehyde (C) [(●) 2 μM; (○) 5 μM; (×) 10 μM; (■) 15 μM], 150 mM potassium phosphate buffer (pH 7.6), EDTA (1 mM), and AdoHcy hydrolase. The mixtures were preincubated at 28 °C, and samples were withdrawn at the indicated times and assayed for residual AdoHcy hydrolase activity as described under Materials and Methods.

Table I: Reversibility of Ribonucleoside 2′,3′-Dialdehyde Induced Inactivation of AdoHcy Hydrolase[a]

ribonucleoside 2′,3′-dialdehyde	treatment of preincubation mixture: KBH_4 reduction[b]	treatment of preincubation mixture: dialysis[c]	% residual activity
			100
AdoHcy dialdehyde (5 μM)			17
AdoHcy dialdehyde (5 μM)		phosphate	28
AdoHcy dialdehyde (50 μM)			0
AdoHcy dialdehyde (50 μM)		Tris	76
AdoHcy dialdehyde (50 μM)	+	Tris	17
adenosine dialdehyde (50 nM)			20
adenosine dialdehyde (50 nM)		phosphate	32
adenosine dialdehyde (100 nM)			16
adenosine dialdehyde (100 nM)		Tris	89
adenosine dialdehyde (100 nM)	+	Tris	22

[a] The standard preincubation mixture consisting of 150 mM potassium phosphate buffer (pH 7.6), EDTA (1.0 mM), and the indicated amounts of inhibitor and AdoHcy hydrolase was incubated at 28 °C for 5 min. Samples, in duplicate, were assayed for enzyme activity at time zero and at the end of the preincubation time and after dialysis. [b] Some samples were treated with KBH_4 (5× concentration of inhibitor) at 28 °C for 2 h and the dialyzed. [c] Some samples were dialyzed directly against 150 mM potassium phosphate buffer (pH 7.6) or first treated with 1.0 M Tris buffer (pH 7.6) containing DTT (5 mM) and EDTA (1.0 mM) at 28 °C for 4.5 h and then dialyzed against the same Tris buffer at 4 °C for 16 h.

inhibitors. When AdoHcy hydrolase was inactivated by any of the three ribonucleoside 2′,3′-dialdehydes, catalytic activity could not be regenerated by prolonged dialysis in phosphate buffer (data for adenosine dialdehyde and AdoHcy dialdehyde are shown in Table I). However, dialysis of these ribonucleoside dialdehyde inactivated enzymes against the nitrogenous buffer Tris resulted in recovery of a substantial amount of enzyme activity (Table I). If, however, the AdoHcy dialdehyde or adenosine dialdehyde inactivated enzymes were pretreated with potassium borohydride and then dialyzed against Tris buffer, enzyme activity was not recovered (Table I).

A characteristic feature of many inhibitors of AdoHcy hydrolase is their oxidation by the enzymatically bound NAD^+ which is subsequently reduced to NADH (Ueland, 1982). With inhibitors such as 2′-deoxyadenosine and 9-β-D-arabinofuranosyladenine, this oxidation is followed by elimination of adenine (Hershfield et al., 1982). However, with adenosine dialdehyde, we did not observe reduction of the enzyme-bound NAD^+ or the appearance of adenine when AdoHcy hydrolase was inactivated by this inhibitor (data not shown).

Protection Studies. The ribonucleoside 2′,3′-dialdehyde induced inactivation of AdoHcy hydrolase could be totally or partially prevented by the inclusion of adenosine, adenine, or AdoHcy in the preincubation mixture (data for AdoHcy dialdehyde are shown in Table II). AMP, ATP, and homocysteine did not protect the enzyme from inactivation. However, homocysteine, at a concentration of 100 μM, caused 50% inhibition of the AdoHcy hydrolase catalyzed hydrolysis of [2,8-^{3}H]AdoHcy (Table II).

Time-Dependent Inactivation of AdoHcy Hydrolase by Ribonucleoside 2′,3′-Dialdehydes. When the hydrolase was incubated with a ribonucleoside 2′,3′-dialdehyde and aliquots were removed at various times and diluted into a solution containing the components of the assay mixture, a time-dependent loss of catalytic activity was observed. The inactivation of AdoHcy hydrolase by the three ribonucleoside 2′,3′-dialdehydes was concentration and time dependent, as seen in Figure 2A–C. It is interesting to note that AdoHcy dialdehyde induced inactivation (Figure 2A) shows pseudo-first-order kinetics at higher concentrations of the inactivator (2 and 5 μM) but becomes curvilinear at the lower concentrations. In contrast, both adenosine dialdehyde (Figure 2B) and MTA dialdehyde (Figure 2C) show curvilinear inactivation of the enzyme at all concentrations. Adenosine dialdehyde is the most potent of the ribonucleoside 2′,3′-dialdehydes, since it was effective at nanomolar concentrations, whereas both AdoHcy dialdehyde and MTA dialdehyde are only effective at micromolar concentrations.

Having established the general characteristics of the interaction of these ribonucleoside 2′,3′-dialdehydes with AdoHcy hydrolase, we decided to focus our attention on one inhibitor for more detailed studies. Since adenosine dialdehyde was the most potent inhibitor and considering its effectiveness in vivo (Bartel & Borchardt, 1984; Keller & Borchardt, 1983), we decided to further characterize the mechanism by which this inhibitor inactivated AdoHcy hydrolase.

Incorporation of [2,5′,8-^{3}H]Adenosine Dialdehyde into AdoHcy Hydrolase. In order to further define the mechanism

Table II: Protection of AdoHcy Hydrolase from Inactivation by AdoHcy Dialdehyde[a]

compd[b]	inhibitor	% residual activity[c]
	+	14
adenosine	+	92
adenine	+	100
AMP	+	20
ATP	+	13
AdoHcy	+	43
L-homocysteine	-	49
L-homocysteine	+	9

[a] The preincubation mixture consisted of 150 mM potassium phosphate buffer (pH 7.6), 1 mM EDTA, the indicated compounds (100 μM), AdoHcy dialdehyde (5 μM), and the enzyme preparation. Samples were withdrawn (395 μL) at 0 and 30 min and assayed for AdoHcy hydrolase activity. The assay mixture consisted of [2,8-^{3}H]-AdoHcy (specific activity 19 μCi/μM), unlabeled AdoHcy (100 μM), and adenosine deaminase (4 units). The incubation time was 10 min at 37 °C. [b] The homocysteine was generated immediately before use by dissolving 15.4 mg (0.1 mmol) of homocysteine thiolactone in 1 mL of 10 mM DTT and 0.3 mL of 1 N KOH. After 10 min at room temperature, the solution was neutralized with 0.3 mL of 1 M KH_2PO_4 and diluted to 5 mL with water to yield a 20 mM solution of homocysteine. [c] The residual activity was calculated with respect to the samples incubated for 0 min.

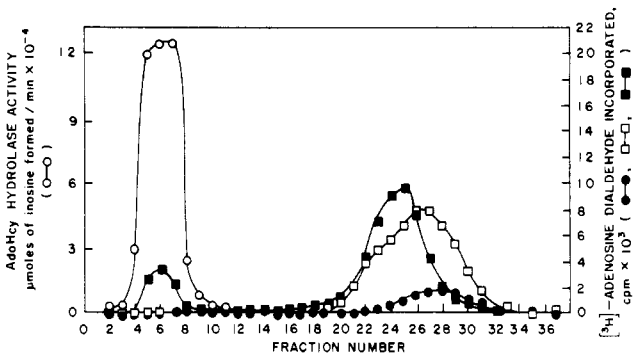

FIGURE 3: Incorporation of [2,5′,8-^{3}H]adenosine dialdehyde into AdoHcy hydrolase. The chromatographic properties of AdoHcy hydrolase were compared to those of the ^{3}H-labeled protein obtained by incubating AdoHcy hydrolase with [2,5′,8-^{3}H]adenosine dialdehyde on a gel filtration column of Bio-Gel P-6 (0.8 × 26 cm). The preincubation mixture consisted of 150 mM potassium phosphate buffer (pH 7.6), EDTA (1 mM), [2,5′,8-^{3}H]adenosine dialdehyde (1 μM) (where appropriate), and AdoHcy hydrolase (0.217 mg) (where appropriate) in a total volume of 2.67 mL. Incubation was carried out at 28 °C for 5 min and then the reaction mixture loaded directly onto the Bio-Gel P-6 column. (○) AdoHcy hydrolase; (□) [2,5′,8-^{3}H]adenosine dialdehyde; (■) AdoHcy hydrolase + [2,5′,8-^{3}H]adenosine dialdehyde; (●) denatured AdoHcy hydrolase + [2,5′,8-^{3}H]adenosine dialdehyde. The column was eluted with 25 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM DTT at 5 °C, and 1-mL fractions were collected and counted. To determine AdoHcy hydrolase activity, aliquots (445 μL) of each fraction were added to assay mixtures consisting of [2,8-^{3}H]AdoHcy (100 μM) and adenosine deaminase (4 units), and hydrolase activity was determined as described under Materials and Methods. To determine the level of ^{3}H-ligand incorporated into the protein, aliquots were simply counted by scintillation spectrometry.

by which the adenosine dialdehyde inactivates AdoHcy hydrolase, we synthesized [2,5′,8-^{3}H]adenosine dialdehyde and studied its interaction with the purified enzyme. Incubation of AdoHcy hydrolase with [2,5′,8-^{3}H]adenosine dialdehyde resulted in the formation of an ^{3}H-labeled protein which had a similar chromatographic pattern as the native enzyme on a Bio-Gel P-6 column (Figure 3). These results suggest the formation of a [2,5′,8-^{3}H]adenosine dialdehyde–AdoHcy hydrolase complex, which is separable from the [2,5′,8-^{3}H]-adenosine dialdehyde by gel filtration. If heat-denatured AdoHcy hydrolase was preincubated with [2,5′,8-^{3}H]adenosine dialdehyde, no radioactivity eluted from the Bio-Gel P-6

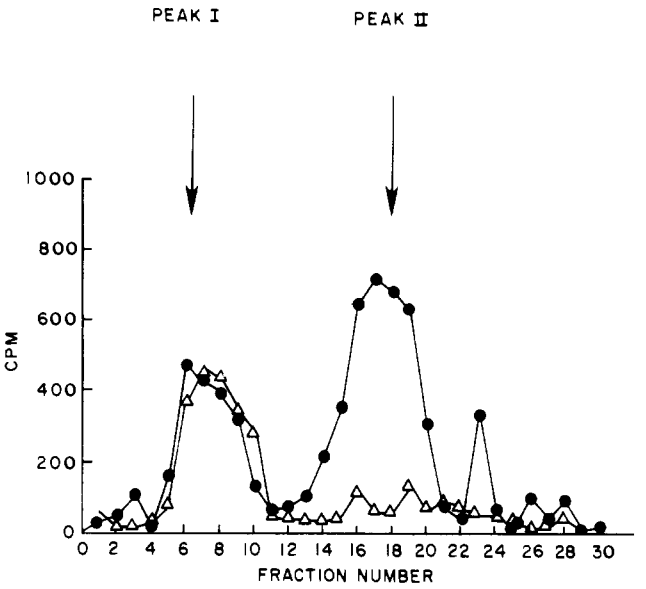

FIGURE 4: Rechromatography of the [2,8-^{3}H]adenosine dialdehyde–AdoHcy hydrolase complex and of the $NaBH_4$-treated [2,8-^{3}H]adenosine dialdehyde–AdoHcy hydrolase complex on Bio-Gel P-6. The ligand–protein complex was formed by incubating (5 min, 28 °C) AdoHcy hydrolase and [2,8-^{3}H]adenosine dialdehyde in buffer as described in the legend to Figure 3. The ligand–enzyme complex was separated from the unreacted ligand by chromatography on a Bio-Gel P-6 column (0.8 × 26 cm) as described in legend to Figure 3. At this stage, the fractions containing the ligand–enzyme complex were pooled, concentrated, then either loaded back onto the same Bio-Gel P-6 column (●) or preincubated with $NaBH_4$ (2.5 μM) for an additional 2 h at 28 °C, and then loaded onto the Bio-Gel P-6 column (△) eluted with 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM DTT at 4 °C. The resulting elution profiles are shown in this figure. The elution patterns of the ligand–enzyme complex alone (peak I) or ligand alone (peak II) are indicated on the figure.

column in the location of the native enzyme or the proposed enzyme–ligand complex (Figure 3). When the substrate adenosine or AdoHcy was included in the preincubation mixtures with AdoHcy hydrolase and [2,5′,8-^{3}H]adenosine dialdehyde, we observed a substantial reduction in the amount of ^{3}H-ligand–enzyme complex formed (data not shown).

To determine the properties of the [2,5′,8-^{3}H]adenosine dialdehyde–AdoHcy hydrolase complex, a sample of the ^{3}H-ligand–enzyme complex was purified free of excess [2,5′,8-^{3}H]adenosine dialdehyde by gel filtration through Bio-Gel P-6. The purified ^{3}H-ligand–enzyme complex was either directly rechromatographed on the Bio-Gel P-6 column or first treated with sodium borohydride and then rechromatographed on the gel filtration column. As shown in Figure 4, chromatography of the purified ^{3}H-ligand–enzyme complex results in partial dissociation of ^{3}H-ligand, thus indicating that the enzyme–ligand complex is dissociable under the conditions used in the chromatography procedure. Although the ^{3}H-ligand dissociated from the enzyme under these conditions, we were unable to detect any enzyme activity in fractions 4–12 (Figure 4). When the purified ^{3}H-ligand–enzyme complex was first treated with sodium borohydride and then chromatographed on a gel filtration column, the ^{3}H-ligand did not dissociate from the complex. Only radioactivity corresponding to the ^{3}H-ligand–enzyme complex was detectable (Figure 4).

When purified AdoHcy hydrolase [specific activity 0.4 μmol of inosine formed min^{-1} (mg of protein)$^{-1}$] was incubated with [2,8-^{3}H]adenosine dialdehyde and the resulting ligand–enzyme complex treated with sodium borohydride and then passed through the gel filtration column, we were able to calculate a stoichiometry of 1.73 nmol of ^{3}H-ligand bound per nmol of

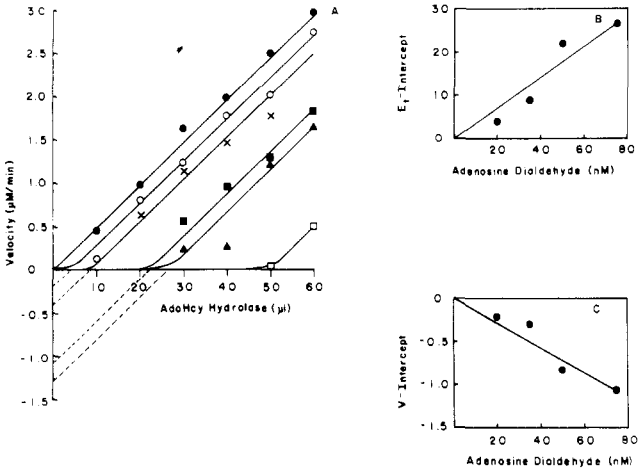

FIGURE 5: Adenosine dialdehyde inactivation of bovine liver AdoHcy hydrolase. (A) Ackermann–Potter plot of enzyme velocity vs. enzyme concentration in the presence of varying concentrations of adenosine dialdehyde. Various concentrations of AdoHcy hydrolase were preincubated with the indicated concentrations of adenosine dialdehyde [(●) 0 nM; (○) 20 nM; (×) 35 nM; (■) 50 nM; (▲) 75 nM; (□) 100 nM] in a total volume of 0.445 mL containing 150 mM potassium phosphate buffer (pH 7.6) and 1 mM EDTA. After preincubation for 5 min at 28 °C, the residual enzyme activity was determined by the addition of 100 μM [2,8-^{3}H]AdoHcy and 4 units of adenosine deaminase, and the mixture was further incubated at 37 °C for 5 min. The reaction was terminated with 100 μL of 5 N formic acid. The product, [2,8-^{3}H]inosine, was then isolated as described under Materials and Methods. (B) Plot of E_t intercept (from Figure 5A) vs. adenosine dialdehyde concentration. From this plot, it can be seen that 10 μL of enzyme corresponds to a concentration of 28 nM. (C) Plot of v intercept (from Figure 5A) vs. adenosine dialdehyde concentration. The slope equals the catalytic value calculated to be 18 min^{-1}.

AdoHcy hydrolase (tetramer).

Kinetic Analysis of Adenosine Dialdehyde Inactivation of AdoHcy Hydrolase. Since adenosine dialdehyde appeared to act as an irreversible or pseudoirreversible inhibitor of AdoHcy hydrolase, we employed the Ackermann–Potter approach (Ackermann & Potter, 1949; Cha et al., 1975) to analyze the interaction of the inhibitor with this enzyme. The Ackermann–Potter approach does not distinguish between irreversible inactivation (e.g., covalent modification where the enzyme is converted to a form which cannot be converted back to the native enzyme) and pseudoirreversible inactivation (e.g., tight-binding inhibitors, where the interaction between the enzyme and the inhibitor is theoretically reversible but where the dissociation constant is so small that, for all practical purposes, the enzyme–inhibitor complex does not undergo dissociation). To study this inhibitor–enzyme interaction, varying amounts of the enzyme were incubated with different concentrations of adenosine dialdehyde for 5 min at 28 °C, after which the residual hydrolase activity was determined by the addition of [2,8-^{3}H]AdoHcy (0.1 mM) and 4 units of adenosine deaminase. Figure 5A is an Ackermann–Potter plot of the resulting data which indicates a stoichiometry of one molecule of adenosine dialdehyde binding to one molecule of AdoHcy hydrolase (tetramer). However, with different enzyme preparations, we have observed up to two molecules of adenosine dialdehyde binding to one molecule of AdoHcy hydrolase (tetramer) (data not shown).

The Ackermann–Potter plot can also be used to determine both the molar equivalency (Figure 5B) and the catalytic turnover (k_3) (Figure 5C) for the enzyme. The calculated molar equivalency of AdoHcy hydrolase (Figure 5B) is 28 nM which is in close agreement with the value of 26.5 nM determined by knowing the protein concentration, enzyme purity,

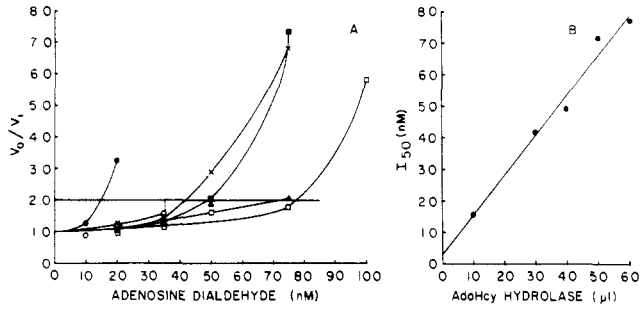

FIGURE 6: Determination of K_i for adenosine dialdehyde inhibition of AdoHcy hydrolase. (A) Plot of V_0/V_i vs. adenosine dialdehyde concentration where V_0 is the velocity in the absence of adenosine dialdehyde and V_i is the velocity in the presence of various amounts of adenosine dialdehyde (from Figure 5A). The V_0/V_i values for different amounts of AdoHcy hydrolase [(●) 10 μL; (○) 20 μL; (×) 30 μL; (■) 40 μL; (▲) 50 μL; (□) 60 μL] were plotted vs. the appropriate inhibitor concentrations. (B) Plot of I_{50} values (from panel A) vs. the amount of AdoHcy hydrolase.

and enzyme molecular weight. The catalytic turnover (k_3) can be calculated in two ways. Knowing the value of S (0.1 mM) and the K_m of AdoHcy (0.01 mM) and also that the slope of the Ackermann–Potter plot is equal to $k_3S/(K_m + S)$, we calculated k_3 = 19 min^{-1}. The constant k_3 can also be estimated from a plot (Figure 5C) of the v-axis intercepts vs. [I_t] which gave a value of 18 min^{-1}.

Although the information obtained from the Ackermann–Potter plot is useful, it cannot be used directly to estimate the inhibition constant, K_i, for adenosine dialdehyde. However, the data can be utilized to determine the I_{50} values (Cha et al., 1975) (the total inhibitor concentration at which the enzyme reaction velocity is 50% of the uninhibited reaction velocity) as seen in Figure 6A. A replot of these I_{50} values vs. enzyme concentrations yields a straight line with an I_{50} intercept equal to 2.39 nm, which represents the K_i value (Figure 6B).

DISCUSSION

The inactivation of AdoHcy hydrolase by various ribonucleoside 2′,3′-dialdehydes, including adenosine dialdehyde, AdoHcy dialdehyde, and MTA dialdehyde, was observed to be both a time-dependent and a concentration-dependent process. The inactivation appears to be irreversible since the enzyme activity could not be recovered after prolonged dialysis against phosphate buffer. However, a substantial percentage of the enzyme activity could be recovered when the inactivated enzyme was dialyzed against a nitrogen buffer, e.g., Tris. Also, this reversal of inhibition could be prevented by pretreatment of the ligand–enzyme complex with sodium borohydride prior to dialysis in Tris buffer. These data suggest that the bond formed between the enzyme and the inhibitor is not irreversible and that it is probably a Schiff base linkage between the aldehydic functionality of the inhibitor and a protein lysinyl residue. The ribonucleoside 2′,3′-dialdehydes appear to bind to the adenosine–AdoHcy site on the enzyme since inclusion of substrates or competitive inhibitors affords protection of the enzyme from inactivation. We did not observe protection with homocysteine. In fact, L-homocysteine itself was found to be an inhibitor of the catalytic reaction in the hydrolytic direction. This observation is consistent with the report that L-homocysteine acts as a mixed-type inhibitor of the hydrolytic reaction (Briske-Anderson & Duerre, 1982).

These data would suggest that the ribonucleoside 2′,3′-dialdehydes fit some of the general criteria as affinity labeling reagents for AdoHcy hydrolase. These generally accepted criteria for an affinity labeling reagent include the following:

(1) a rate saturation effect on the inactivation of the enzyme by the inhibitor; (2) protection of the enzyme by substrates or competitive inhibitors against inactivation; and (3) stoichiometric incorporation of one molecule of the inhibitor per binding site. However, there exist at least three features of the ribonucleoside 2′,3′-dialdehyde–AdoHcy hydrolase reaction that are not consistent with a true affinity labeling mechanism: (1) the reversibility by Tris buffer of the inactivation of AdoHcy hydrolase by ribonucleoside dialdehydes (Table I); (2) the dissociation of [2,8-^{3}H]adenosine dialdehyde from the ligand–enzyme complex during gel filtration (Figure 4); and (3) the observation that in L-929 mouse cells treated with adenosine dialdehyde the AdoHcy hydrolase activity returns to the control value within 16 h and this restoration of AdoHcy hydrolase activity is not due to synthesis of new protein, since activity returned even in the presence of cycloheximide, a protein synthesis inhibitor (Bartel & Borchardt, 1984). These observations strongly suggest that the bond formed between AdoHcy hydrolase and adenosine dialdehyde is not irreversible and, hence, the inactivation of AdoHcy hydrolase by ribonucleoside dialdehydes is better described as pseudoirreversible.

Our analysis of the kinetics of inactivation of AdoHcy hydrolase would further support our conclusion that these ribonucleoside dialdehydes are pseudoirreversible inhibitors and best characterized as tight-binding inhibitors. From the Ackermann–Potter analysis of the inactivation data (Figure 5A), it is apparent that adenosine dialdehyde acts as a tight-binding inhibitor of AdoHcy hydrolase with one molecule of adenosine dialdehyde binding per tetramer of enzyme. In contrast, with some enzyme preparations, we have observed up to two molecules of adenosine dialdehyde binding per tetramer of enzyme (data not shown). Furthermore, when we studied a series of carbocyclic purine nucleoside 2′,3′-dialdehydes (Houston et al., 1985) as inhibitors of AdoHcy hydrolase, we observed 2:1, 3:1, and up to 4:1 complexes of these compounds with the enzyme. This variability in the stoichiometry may be due to the chemical reactivity of these dialdehydes and/or the fact that AdoHcy hydrolase has two nonequivalent adenosine binding sites (Abeles et al., 1982).

To determine the K_i for adenosine dialdehyde, the I_{50} values were determined (Figure 6A) at various different concentrations of the enzyme. A plot of the I_{50} values vs. enzyme concentrations resulted in a straight line with the I_{50} intercept at 2.39 nM (Figure 6B) which is the K_i for adenosine dialdehyde. This value is similar to the value (3.3 nM) reported earlier by Hoffman (1979).

In comparison to other reported inhibitors of AdoHcy hydrolase, our results indicate that adenosine dialdehyde is significantly more potent than 3-deazaadenosine (K_i = 4 μM) (Chiang et al., 1977), 9-β-D-arabinofuranosyladenine (K_i = 5 μM) (Hershfield, 1979), and 2′-deoxyadenosine (K_i = 66 μM compared to K_i = 24 μM for 9-β-D-arabinofuranosyladenine) (Hershfield, 1979), the latter two of which also cause irreversible inactivation of the enzyme. However, the inhibitory activity of adenosine dialdehyde is quite comparable to that of aristeromycin [K_i = 5 nM (Guranowski et al., 1981), K_i = 110 nM (Houston et al., 1985)], 3-deazaaristeromycin [K_i = 3 μM (Montgomery et al., 1982), K_i = 4 nM (Houston et al., 1985)], and neplanocin A (K_i = 8.39 nM) (Borchardt et al., 1984).

In summary, these data clearly show that adenosine dialdehyde exhibits the properties of a pseudoirreversible or tight-binding-type inhibitor of purified bovine liver AdoHcy hydrolase. This mechanism would explain the prolonged inhibitory effects (up to 72 h) of adenosine dialdehyde on AdoHcy hydrolase in 929 mouse L cells in culture (Bartel & Borchardt, 1984). The inhibitory effects of adenosine dialdehyde on AdoHcy hydrolase and subsequent increases of cellular AdoHcy levels and inhibition of crucial AdoMet-dependent methylations (e.g., mRNA methylation) are the likely mechanisms by which this agent inhibits vaccinia viral multiplication in 929 mouse L cells (Keller & Borchardt, 1983).

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Effect of Monovalent Cations on the Pre-Steady-State Kinetic Parameters of the Plasma Protease Bovine Activated Protein C†

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ABSTRACT: Activated bovine plasma protein C (APC) was not reactive with the substrate *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) in the absence of cations. In the presence of increasing concentrations of Na^+, the acylation rate constant, $k_{2,app}$, at 7 °C, progressively increased from 0.32 ± 0.03 s^{-1} at 12.5 mM Na^+ to 1.15 ± 0.10 s^{-1} at 62.5 mM Na^+. A linear dependence of the reciprocal of $k_{2,app}$ with $[Na^+]^{-2}$ was observed, indicating that at least two monovalent cation sites, or classes of sites, are necessary for the catalytic event to occur. From this latter plot, the $k_{2,max}$ for APC catalysis of NPGB hydrolysis, at saturating $[Na^+]$ and [NPGB], was calculated to be 1.21 ± 0.10 s^{-1}, and the K_m for Na^+ was found to be 21 ± 3 mM. The dissociation constant, K_s, for NPGB to APC, at 7 °C, was not altered as $[Na^+]$ was increased, yielding a range of values of 18.5 × 10^{-5} to 19.9 × 10^{-5} M as $[Na^+]$ was varied from 12.5 to 62.5 mM. The deacylation rate constant, k_3, for *p*-guanidinobenzoyl-APC hydrolysis was also independent of $[Na^+]$, with a value of (3.8 ± 1.0) × 10^{-3} s^{-1} in the absence of Na^+ or in the presence of concentrations of Na^+ up to 200 mM. Identical kinetic behavior was observed when Cs^+ was substituted for Na^+ in the above enzymic reaction. The pre-steady-state kinetic parameters were calculated according to the same methodology as described above. The $k_{2,max}$ for acylation by NPGB was found to be 1.36 ± 0.10 s^{-1}, the K_m for Cs^+ was 12 ± 2 mM, the K_s for NPGB was (1.8–2.2) × 10^{-5} M, and the k_3 for deacylation of *p*-guanidinobenzoyl-APC was determined to be (3.8 ± 1.0) × 10^{-3} s^{-1}.

Activated protein C (APC) is a serine protease which is formed by limited proteolytic hydrolysis of its precursor molecule, protein C (PC). Protein C has been purified from bovine (Stenflo, 1976) and human (Kisiel, 1979) plasmas and has been shown to be one of the vitamin K dependent plasma proteins (Stenflo, 1976). After purification, bovine PC has been found to consist of two polypeptide chains of total molecular weight of approximately 56000 per molecule. Its light chain contains 155 amino acid residues of known sequence and one site of glycosylation which exists at Asn_{97} (Fernlund & Stenflo, 1982). All 11 residues of Gla exist within the amino-terminal 35 residues of this chain (Fernlund & Stenflo, 1982), and a single residue of *erythro*-β-hydroxyaspartic acid has been identified herein at position 71 (Drakenberg et al., 1983). The heavy chain of bovine plasma PC contains 260 amino acid residues of known sequence, and three sites of glycosylation are present, at Asn residues 93, 154, and 170 (Stenflo & Fernlund, 1982). This chain contains the latent active-site serine residue of APC, at position 201, with the charge-relay system most likely comprising His_{56}, Asp_{102}, and Ser_{201} (Stenflo & Fernlund, 1982). The heavy and light chains of bovine PC and APC are linked by a single disulfide bond consisting of Cys_{122} in the heavy chain (Stenflo & Fernlund, 1982) bound to a yet nonidentified Cys residue in the light chain.

The activation of PC to APC is accompanied by release of a 14-residue polypeptide from the amino terminus of the heavy chain of bovine PC (Kisiel et al., 1976) and a 12-residue polypeptide from the same location in human PC (Kisiel, 1979). The enzymes capable of catalyzing cleavage of the requisite peptide bond include α-thrombin (Kisiel et al., 1977), trypsin (Kisiel et al., 1976), and the factor X activating enzyme from the venom of Russell's viper (Kisiel et al., 1976).

APC functions as an anticoagulant, most probably by nature of its ability to inactivate, by proteolysis, a cofactor, factor Va, necessary for prothrombin activation (Kisiel et al., 1977) and, similarly, a cofactor, factor VIIIa, necessary for factor X activation (Vehar & Davie, 1980). Bovine APC catalyzes the hydrolysis of a number of synthetic ester (Steiner et al., 1980) and amide (Kisiel et al., 1976; 1977; Steiner & Castellino, 1982) substrates. This enzyme is virtually unique as a protease in that monovalent and/or divalent cations are necessary in order for APC to exert activity toward small substrates (Steiner et al., 1980; Steiner & Castellino, 1982, 1985). We are greatly interested in identifying the enzymic properties of APC which are influenced by such cations and have examined in this report the pre-steady-state kinetics of APC toward a synthetic substrate and the influence of mo-

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