J. Enzyme Inhibition, 1995, Vol. 8, pp. 243–253
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Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/16/11
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COMPARISON OF THE INHIBITION OF TYPE A AND TYPE B *S*-ADENOSYLHOMOCYSTEINE HYDROLASE: EFFECTS OF COFACTOR CONTENT ON INHIBITION BEHAVIOR AND NUCLEOSIDE BINDING

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(Received 30 August 1994; in final form 8 November 1994)

The enzyme *S*-adenosylhomocysteine hydrolase (E.C.3.3.1.1) occurs in two forms in bovine liver: Type A, which carries four moles of NAD^+ per mole of enzyme tetramer, and Type B, which carries two moles of NAD^+ per mole of tetramer.[1] The inhibition of these two forms of the enzyme with 2',2'-difluoro-2'-deoxyadenosine has been investigated. The studies examined the binding stoichiometry and stability of the enzyme-inhibitor complexes formed from each type of the enzyme, the degree of NAD^+ reduction and NAD^+ release, and the possibility of covalent bond formation between the enzyme and the inhibitor. Significant differences in the behavior of the two forms of the enzyme were encountered which may have important implications for the design of *S*-adenosylhomocysteine hydrolase inhibitors as therapeutic agents.

KEY WORDS: *S*-Adenosylhomocysteine hydrolase, time-dependent inhibition, fluorine-containing adenosine analogs

INTRODUCTION

The enzyme *S*-adenosylhomocysteine hydrolase (SAH hydrolase) (E.C.3.3.1.1) catalyzes the reversible hydrolysis of *S*-adenosylhomocysteine (**1**) (SAH) to adenosine (**2**) and homocysteine (**3**) in the absence of exogeneous cofactors (Equation 1). SAH hydrolase was first isolated from rat liver[2] and subsequently found to be widespread in eucaryotes.[3–11] Occurrence in procaryotes has also been reported.[12] The mammalian enzyme is tetrameric, with a molecular weight of ca. 190,000, and it usually carries 1 molecule of nucleotide cofactor per subunit. However, the bovine liver enzyme appears to occur in two forms: Type A, which carries four cofactor molecules per enzyme tetramer,[1,7] and Type B, which contains only two equivalents of cofactor

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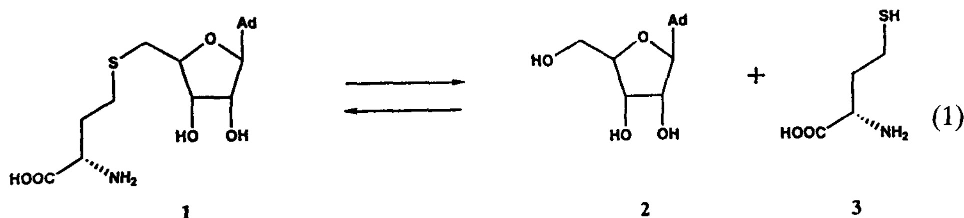
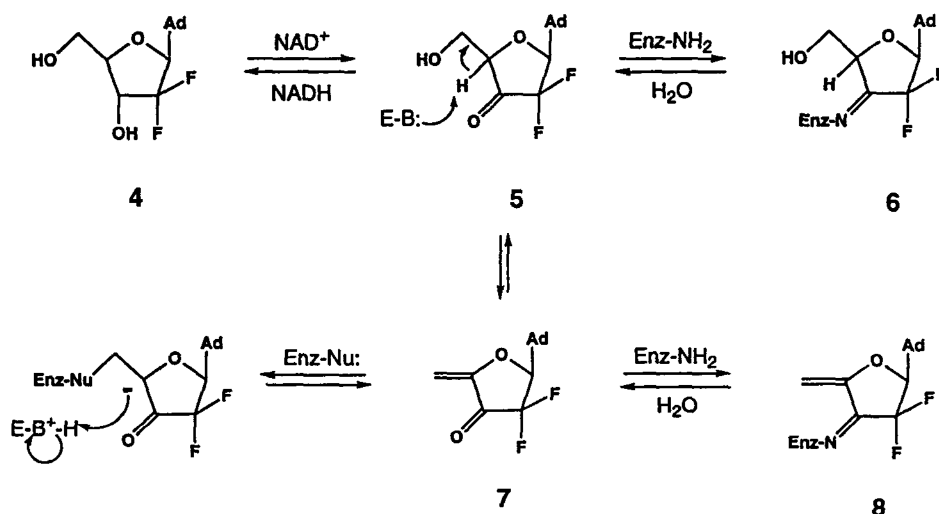


per tetramer.[1,13] Between 10 and 50% of the bound cofactors can be present in reduced form, regardless of the total cofactor content of the enzyme.[11,13,14]

Mechanistic studies of the bovine liver enzyme have shown that the reaction catalyzed by the enzyme is a multistep process.[7] Enzyme-bound NAD^+ oxidizes the 3'-hydroxyl group of adenosine to give the 3'-keto derivative which then undergoes β-elimination to yield 3'-keto-4',5'-didehydro-5'-deoxyadenosine. Michael addition of water to the latter compound then forms 3'-ketoadenosine, which is finally reduced to adenosine by enzyme-bound NADH.

Considerable attention has been focused on pharmacological agents that will inhibit SAH hydrolase. SAH is a potent inhibitor of all *S*-adenosylmethionine dependent methyl transferases. As a consequence, many inhibitors of SAH hydrolase exhibit antiviral[15–19] and antiretroviral activity[17] and there is a direct correlation between the potency of the inhibitor and its antiviral activity.[20] Recently, inhibitors of *S*-adenosylhomocysteine hydrolase have also been proposed as potential antiparasitic agents which would act by selective inhibition of the parasite enzyme relative to the mammalian enzyme.[21]



SCHEME 1 Hypothetical mechanism of inactivation of *S*-adenosylhomocysteine hydrolase by 2'-deoxy-2',2'-difluoroadenosine (**4**).

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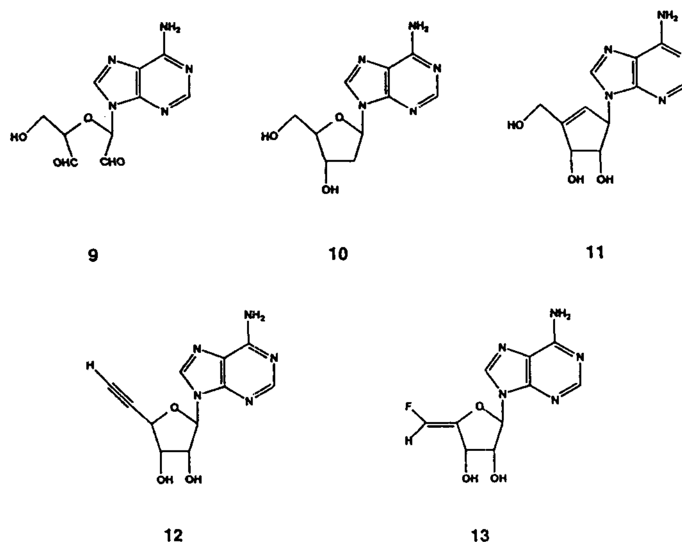

FIGURE 1 Inhibitors of *S*-adenosylhomocysteine hydrolase.

2',2',-Difluoro-2'-deoxyadenosine (dFdAdo) (**4**) (Scheme 1) has been found to exhibit potent cytotoxicity toward some human leukemia cell lines.[22] We have therefore decided to investigate the inhibition of SAH hydrolase by dFdAdo in order to determine if the interesting biological activity displayed by this compound could be partly due to its interaction with SAH hydrolase.

A second rational for the examination of dFdAdo as an inhibitor for SAHase is based upon earlier investigations carried out with adenosine dialdehyde[23] (**9**) and with 2'-deoxyadenosine[24] (**10**) (Figure 1). The presence of the 2',2'-difluoro functionality should assure the stability of the proposed 3'-keto intermediate and prevent the cleavage of the glycosidic linkage which occurs with **10**. Furthermore, the keto group of **5** will be highly electrophilic and it should readily form a Schiff base linkage with the putative lysine residue suggested to be present at the active site of the enzyme by the studies with adenosine dialdehyde. The proposed mechanism for the fluoroinhibitor is outlined in Scheme 1. The enzyme should oxidize the 3'-hydroxy group of **4** to form a 3'-keto intermediate **5** which could then undergo either formation of a Schiff base linkage with a lysine residue at the active site of the enzyme to form **6** or elimination of water to form 4',5'-didehydro-3'-keto-2'-deoxy-2',2'-difluoroadenosine (**7**). The latter compound could then undergo nucleophilic attack at C-5' by an enzyme nucleophile or form a Schiff base with the 3'-keto group resulting in the enzyme-inhibitor complex **8**.

Another reason to examine the interaction of dFdAdo with SAH hydrolase is derived from previous studies which have suggested that inhibitor-sensitive and inhibitor-insensitive forms of SAH hydrolase coexist in various cell lines.[25-27] These studies revealed that even the most potent SAH hydrolase inhibitors only produce

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about 80–85% inhibition of the enzyme.[25,26,28] Furthermore, the antiviral effects of a given inhibitor were found to vary up to 200-fold, depending on the nature of the cultured host cells.[29] In order to explain these observations, it has been postulated that different forms of the enzyme exist. However, the nature of the differences remains unknown. Consequently, it was of interest to investigate whether the difference in the nucleotide cofactor content of Type A and Type B SAH hydrolase would lead to differences in inhibition behavior with dFdAdo.

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MATERIALS AND METHODS

[2-^{3}H]-2',2'-Difluoro-2'deoxyadenosine was a gift from Dr. William Plunkett of the M.D. Anderson Hospital and Tumor Institute at Houston. All other tritium labeled compounds were obtained from Amersham Corporation and from New England Nuclear. All other reagents were purchased from Sigma, Aldrich Chemical Company or Fluka and were used without further purification. [2-^{3}H]-*S*-adenosyl-L-homocysteine was prepared as previously described.[1] Isolation of *S*-adenosylhomocysteine hydrolase from bovine liver was carried out in the manner previously described.[1] SAH hydrolase activity was assayed in the hydrolytic direction by the Narayanan and Borchardt modification[13] of the method of Richards *et al.*[6] using [2-^{3}H]-*S*-adenosylhomocysteine,[6] or by using the spectrophotometric procedure of Palmer and Abeles[7] in combination with unlabeled *S*-adenosylhomocysteine. Protein concentrations were determined by the method of Bradford[30] using bovine serum albumin as a standard. The inhibition constant and the catalytic turnover number for mechanism-based inactivation were termed K_I and k_{inact}, respectively, in order to avoid confusion with the constants for reversible inhibitors (K_i, k_{cat}).[31]

Kinetic Studies

In the time-dependent inactivation study, SAH hydrolase (19.8 μL, 21.6 μg, Type A enzyme or 3.2 μL, 36 μg, Type B enzyme) was preincubated in 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM of dithiothreitol and EDTA for 5 min at 37°C in a shaking water bath. Various amounts of dFdAdo were added to a final concentration of 667 μM, 333 μM, 166 μM, 83 μM and 42 μM in a total volume of 150 μL. The sample was then vortexed for 1 s and immediately transferred to the water bath. Aliquots of the incubation mixture were removed after 10 min, 30 min, 90 min, 2.5 h, and 4 h and assayed for residual enzyme activity by use of the radioactive assay.

The K_I and k_{inact} were determined using the method of Kitz and Wilson.[32] SAH hydrolase (18 μg, 16.5 μL, Type A enzyme or 2.7 μL, 30 μg, Type B enzyme) was preincubated with 20 mM potassium phosphate buffer, pH 7.4 for 10 min at 37°C. Various amounts of dFdAdo were added to obtain a final concentration of 400 μM, 240 μM, 160 μM, 120 μM, and 96 μM in the case of the Type A enzyme and 800 μM, 400 μM, 240 μM, or 120 μM in the case of the Type B enzyme. The total volume was 125 μL. Enzyme aliquots were removed after 4, 10, 16 and 22 min and the residual enzyme activity was determined with the radioactive assay.

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The protection of SAH hydrolase by adenosine was determined by incubating SAH hydrolase with dFdAdo and various amounts of adenosine (**2**) which were added in a ratio of 1:10, 1:200, 1:1000 of substrate to inhibitor. The enzyme (Type B, 2.7 μL, 30 μg) was preincubated in varying amounts of 20 mM potassium phosphate buffer, pH 7.4 at 37°C in a shaking water bath. At t = 0, 20 μL of dFdAdo (2 mg/mL) and different amounts of adenosine (2 mg/mL) were added. The total reaction volume was 150 μL in all cases. Aliquots were removed every 8 min over a period of 32 min and the residual enzyme activity was determined using the assay that employs [2-^{3}H]-*S*-adenosylhomocysteine.

Labeling Studies with [2-^{3}H]dFdAdo

SAH hydrolase (13.6 μL, 100 μg) was incubated in 26.5 μL of 50 mM potassium phosphate buffer, pH 7.6, containing 1 mM of dithiothreitol and EDTA and [2-^{3}H]dFdAdo (79.9 μL, 2 mg/mL, 0.56 μmole, sp. act. 9.22 μCi/mmole) was added. The reaction mixture was incubated for 4 h at 37°C and subsequently dialyzed against 200 mL of 20 mM potassium phosphate buffer, pH 7.4, over 36 h with 4 changes of buffer. The amount of recovered protein was determined, its radioactivity was measured, and the binding stoichiometry was then calculated.

In order to determine the degree of irreversible inactivation of the labelled enzyme-inhibitor complex, the enzyme-dFdAdo adduct was synthesized as described above and subsequently dialyzed exhaustively against 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM of dithiothreitol and EDTA. The specific enzyme activity was determined at t = 0 and 3 h and after dialysis. The activities were corrected with a control containing no inhibitor.

Stabilization of the enzyme-dFdAdo complex was attempted by sodium borohydride reduction. SAH hydrolase (19.9 μL, 150 μg) was incubated with dFdAdo (95.6 μL, 10 mg/mL) in 50 μL 0.5 M potassium phosphate buffer, pH 7.4 for 90 min at 37°C. $NaBH_4$ (50 mg/mL 50 mM NaOH) was added in 0.75 μL portions over a period of 45 min every 5 min, followed by 1.5 μL every 10 min for 40 min. The reaction was allowed to proceed overnight, and the protein was then exhaustively dialyzed against 200 mL of 20 mM potassium phosphate buffer, pH 7.4 for 2 days with 5 buffer changes.

The stability of the enzyme-dFdAdo complex in its reduced or nonreduced form was determined with a variety of denaturation conditions. Enzyme-inhibitor complex, synthesized as outlined above, was denatured with either 20 mM potassium phosphate buffer, pH 7.4, containing 8 M urea or a solution of 1% SDS and 0.1% TFA in water, pH 2.3–2.4 for 1–3 days at RT. The binding stoichiometry after denaturation was determined as before.

Reactivation of the Type B-enzyme-inhibitor-dFdAdo complex was accomplished upon incubation with exogeneous NAD^+. SAH hydrolase (22.5 μg, 21 μL) was preincubated with 40 μL of 20 mM potassium phosphate buffer, pH 7.4 for 10 min at 37°C. dFdAdo (10.2 μL, 2 mg/mL) was added and the reaction mixture was allowed to incubate at 37°C for 2.5 h. NAD^+ (66.2 μL, 200 mg/mL in 20 mM potassium phosphate buffer, pH 7.4, 18.7 μM) was added and the reaction was continued for 3.5 h at 25°C. The enzyme activity was measured before the addition of the inhibitor and after 2.5 h and 6 h.

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Determination of the NAD$^+$ and NADH Content of the Enzyme-dFdAdo Complex[33]

SAH hydrolase (74.8 μL, 800 μg) was incubated with dFdAdo (617 μL, 2 mg/mL in 20 mM potassium phosphate buffer, pH 7.4) for 2.5 h at 37°C. For determination of the total NAD$^+$ content, the sample was lyophilized, redissolved in 40 μL of 20 mM potassium phosphate buffer, pH 7.0, treated with 1 N perchloric acid to a final concentration of 0.2 M, and the mixture incubated for 5 min at 4°C. It was then neutralized with 1 N KOH and centrifuged. The supernatant was removed and the precipitate was washed two additional times with 20 μL of H_2O. All the fractions were combined and used in the following NAD$^+$ assay. Glycine HCl (0.4 mL of a 0.25 M solution, containing 0.9 mM glutathione) was mixed with 0.4 mL of a 46.5 mM semicarbazide hydrochloride solution, pH 7.0, and 40 μL of 95% EtOH in a 1 mL cuvette. The NAD$^+$ containing enzyme solution (100 μL) was added. The solution was mixed well and used as the blank to measure background absorption at 340 nm. Horse liver alcohol dehydrogenase (0.08 mg, 8 μL) was added to start the reaction and the mixture was incubated for 15 min at room temperature. The absorbance at 340 nm was determined and the absolute NAD$^+$ content was calculated with the help of a prepared standard curve (2–10 nm NAD$^+$). The per cent recovery for NAD$^+$ from this assay was determined by using a known amount of NAD$^+$ and treating it in an identical fashion as the enzyme sample. This corrects for the nucleotide cofactor which is destroyed or lost during the denaturation and subsequent separation from the enzyme. In a duplicate run, an average recovery of 72% was determined.

In order to measure the total amount of NAD$^+$ released during inactivation, the reaction mixture was filtered through a 10,000 MW cutoff filter unit and the filter was washed with 50 μL of water. The filtrates were combined and lyophilized to dryness. The sample was redissolved in 100 μL of 20 mM potassium phosphate buffer, centrifuged to remove insoluble components and used directly for the coupled UV assay.

Analysis of Reaction Products

The presence of a reactive species prior to inactivation was investigated by preincubating Type B SAH hydrolase (5.4 μL, 11.04 mg/mL) in 20 mM potassium phosphate buffer, pH 7.4 for 10 min at 37°C. At t = 0, dFdAdo (60 μL, 2 mg/mL) was added and the degree of inactivation by dFdAdo was monitored every 8 min over a 64 min period. Enzyme aliquots were removed and the activity was determined with the radioactive assay. After 32 min, a fresh equivalent of the enzyme (5.4 μL, 11.04 mg/mL) was added. The measured decrease of residual activity of the second enzyme equivalent was corrected with a control having no additional enzyme added.

In order to detect any SAH-like products, dithiothreitol-free SAH hydrolase (Type A) was prepared by exhaustive dialysis against 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA. Subsequently, the dithiothreitol-free enzyme (35.7 μL, 125 μg) was diluted with 38 μL of the dialysis buffer and subsequently incubated with labeled (26.7 μL, 3 μCi) and unlabeled (6 μL, 2 mg/mL, 42 μmole) dFdAdo, and 16.8 μL of DL-homocysteine (10 mmole/mL) for 16 h at 37°C. The sample was filtered through an Amicon ultrafiltration unit having a 10,000 MW cutoff

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to remove the protein and the filtrate was lyophilized to dryness. The residue was redissolved in 20 μL of water and chromatographed on a cellulose plate using ethanol, water, and acetic acid in a ratio of 65:34:1. In this solvent system, the R_f-values for SAH, DL-homocysteine, and dFdAdo were 0.26, 0.59, and 0.78, respectively. The compounds were visualized by UV, ninhydrin, and radioscanning. Radioscanning of thin-layer chromatograms was carried out using a Berthold LB 2832 TLC Linear Analyzer. A control incubation which contained no homocysteine was also carried out.

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RESULTS AND DISCUSSION

The protocol which we employ for the isolation and purification of bovine SAH hydrolase usually leads to the isolation of two forms of the enzyme that we have termed Type A and Type B.[1] Type A contains four moles of nucleotide cofactor per mole of enzyme tetramer, while Type B contains only two moles per mole of tetramer. These two forms of the enzyme are resolved during chromatography on blue-dextran agarose. The Type B enzyme has been previously isolated from bovine liver in the apparent absence of Type A enzyme by blue dextran agarose chromatography.[13] We have now confirmed this observation since one out of five isolations that we carried out yielded the Type B enzyme exclusively. This clearly indicates that the proportion of Type A to Type B in bovine liver varies significantly from animal to animal. This finding is especially significant since these two forms are not interconvertible *in vitro*.[1] The Type A enzyme employed in the experiments discussed below contained four moles of NAD^+ per mole of enzyme tetramer. Type B enzyme utilized for the mechanistic studies was not exposed to adenosine during the isolation procedure and it contained 2 moles of NAD^+ per mole of enzyme tetramer.

The adenosine analog dFdAdo was shown to inactivate both forms of SAH hydrolase in a concentration and time-dependent manner. The inhibition constant K_I and the catalytic turnover number k_{inact} were determined by the method of Kitz and Wilson[32] for both the Type A enzyme and Type B enzyme. A K_I value of 694 μM and a k_{inact} of 0.11 min^{-1} were obtained for Type A enzyme. In the case of Type B enzyme, a K_I of 250 μM and a k_{inact} of 0.08 min^{-1} were determined, indicating a nearly 3-fold difference in potency. We confirmed that the action of dFdAdo is active site directed by means of a protection experiment[31] with adenosine using Type B SAH hydrolase. A large excess of the inhibitor (1000 fold) was required to obtain a degree of enzyme inhibition similar to that observed in the absence of adenosine.

The inactivation of Type A SAH hydrolase by dFdAdo coincided with the irreversible binding of ca. 3 moles of inhibitor per mole of enzyme tetramer, the reduction of one mole of NAD^+ to NADH, and the release of ca. 1–2 moles of enzyme-bound NAD^+ into the medium. This behavior is somewhat similar to the closest analog of dFdAdo, 2'-deoxyadenosine (**10**).[14,24] The latter compound inactivates Type A SAH hydrolase with the irreversible binding of 2–4 moles of inhibitor, the reduction of 2 moles of NAD^+, and the release of the remaining NAD^+ from the enzyme. However, in contrast to 2'-deoxyadenosine, the inhibition of Type A SAH hydrolase

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by dFdAdo could be partly reversed by exhaustive dialysis, since the dialyzed enzyme exhibited activity corresponding to 50 to 60% of that for the fully active enzyme. The observed reactivation of the enzyme-dFdA complex after dialysis can be explained if one assumes that a fourth equivalent of the inhibitor binds in an reversible fashion and that the loss of this fourth equivalent upon dialysis leaves the enzyme with one active subunit. Another difference between the inactivation of Type A SAH hydrolase by dFdA and by **10** is that the oxidized form of the latter compound undergoes β-elimination of adenine within the active site of the enzyme.[24] Such a reaction path is precluded for the oxidized form of dFdA since no hydrogens are present at C-2'.

Surprisingly, the inactivation of Type B SAH hydrolase by dFdAdo was associated with the irreversible binding of only ca. one mole of inhibitor per mole of enzyme tetramer, the reduction of ca. one mole of NAD^+ to NADH, and the release of the remaining one equivalent of NAD^+ from the enzyme tetramer. This behavior is somewhat unusual when compared to that of some other well-studied inhibitors of SAH hydrolase. In the case of neplanocin A[34] (**11**), acetylenic adenosine[1] (**12**), and (*E*)-5'-deoxy-4'-5'-didehydro-5'-fluoroadenosine[35] (**13**) (Figure 1), there is a 1:1 relationship between the number of equivalents of inhibitor that are bound in an irreversible fashion and the sum of the number of equivalents of NAD^+ reduced or released from the enzyme. The unusual behavior exhibited in the interaction of dFdAdo with Type B SAH hydrolase can be explained if a second equivalent of dFdAdo binds to the enzyme in a reversible fashion causing the release of one mole of NAD^+ per mole of enzyme tetramer. The second equivalent would then be removed by exhaustive dialysis. An alternative explanation for the unusual behavior observed with dFdAdo would involve an interaction between the subunits of the tetrameric complex. In this case, the binding of a single mole of inhibitor per mole of enzyme tetramer would not only promote the reduction of one mole of NAD^+ to NADH but also induce some type of conformational change which results in the release of NAD^+ from a different subunit of the same complex.

Incubation experiments demonstrated that saturating concentrations of the dF-dAdo can reduce the activity of both types of SAH hydrolase to a maximum of ca. 15% of the original value even after prolonged incubation for 4–6 h. Reactivation of the Type A enzyme occurred on exhaustive dialysis. In contrast to the Type A enzyme, the complex formed between dFdAdo and Type B SAH hydrolase could not be reactivated, even after exhaustive dialysis. This behavior can be explained by the fact that the Type B SAH hydrolase-inhibitor complex contains no bound NAD^+ after complete inactivation. Support for this explanation is provided by the observation that incubation of the complex with saturating concentrations of exogenous NAD^+ completely restores the activity of the enzyme. Similar behavior has been reported for the inactivated complexes formed between the Type B enzyme, neplanocin A[34] (**11**) and neplanocin A analogs.[36]

Based on our proposed mechanism of inactivation, the oxidation of dFdAdo with concurrent reduction of NAD^+ to NADH leads to the formation of **5** and/or **7** (Scheme 1) which should be sufficiently electrophilic to form a covalent linkage with an active site nucleophile. In order to study this putative covalent enzyme-inhibitor complex in more detail, its stability under a variety of denaturation conditions

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was determined. When the complex between Type A enzyme and [2-^{3}H]-dFdAdo prepared under standard conditions was denatured with 8 M urea at 4°C for 1–3 days, 0.6 eq of inhibitor remained bound per mole of enzyme tetramer, while harsher conditions using 8 M urea or 1% SDS/0.1% TFA, pH 2.3 at room temperature for 1–3 days led to the release of all the labelled material from the enzyme. We therefore attempted to stabilize the putative dFdAdo-Type A enzyme linkage by borohydride reduction. Indeed, increased stability of the labelled enzyme-inhibitor adduct against denaturation under all conditions used was observed. In the case of the 8 M urea denaturation at 4°C, 2 eq remain bound to the enzyme, while with 8 M urea or SDS/TFA at room temperature, 0.5 eq remain bound. The fact that approximately one equivalent of the inhibitor was more tightly bound after reduction is consistent with the formation of only one equivalent of NADH during inactivation of the enzyme.

Since it appeared that a similar mechanism of covalent bond formation could exist for Type B enzyme, the effect of borohydride reduction on the stability of the Type B-dFdAdo complex was examined. However, all attempts to stabilize the Type B enzyme-inhibitor linkage were unsuccessful. The denaturation of the reduced enzyme-inhibitor complex with either 8 M urea or SDS/TFA released all the labelled material from the enzyme. This result stands in contrast to the behavior exhibited by the Type A-dFdAdo complex, and it may indicate that no covalent bond is formed between the Type B enzyme and the oxidized form of dFdAdo. The reason for this difference in behavior between the complexes formed by dFdAdo with the two types of SAH hydrolase is unclear.

In order to examine the mechanism of inactivation by dFdAdo in more detail, the possibility that a reactive species is formed from dFdAdo and released prior to inactivation was investigated. If this mechanism of inactivation were correct, then a time-dependent increase of the rate of inactivation would be observed due to the build-up of the reactive species in solution. Therefore, if a second equivalent of fresh enzyme is added to the incubation mixture, it should be inactivated at a higher rate than that of the first equivalent of the enzyme. This is based on the assumption that the reactive species is stable in solution and is not immediately quenched by reaction with a nucleophile. In the case of Type B SAH hydrolase, the rate and degree of inactivation of the first and second equivalents of enzyme were found to be similar. Consequently, the formation of a reactive species prior to inactivation is unlikely. This is further supported by the observation that no reaction products were detected in solution upon incubation of dFdAdo with the enzyme in the absence of homocysteine. Furthermore, no SAH-like product was formed from dFdAdo in the presence of homocysteine (**3**), indicating that dFdAdo does not serve as a substrate for the enzyme. The major features of the inhibition of Type A and Type B SAH hydrolase by dFdAdo are outlined in Table 1.

In summary, the results of the investigations of the inhibition of the two types of bovine SAH hydrolase by 2', 2'-difluoro-2'-deoxyadenosine has revealed that the two forms of the enzyme exhibit significant differences in behavior. These observations indicate that caution should be exercised when attempting to predict the inhibition behavior that will be exhibited by one form of the enzyme on the basis of the inhibition behavior exhibited by the other form. Our results also suggest that the variations

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TABLE 1
Behavior of Type A and Type B SAH Hydrolase Upon Treatment with dFdAdo (**4**)

	Type A	Type B
K_I	694 μM	250 μM
k_{inact}	0.11 min^{-1}	0.08 min^{-1}
Degree of inactivation	85%	85%
Degree of reactivation upon dialysis	50–60%	no reactivation
Binding stoichiometry (eq inhibitor per tetramer)	ca. 3	ca. 1
Eq of NAD^+ reduced	1	1
Eq of NAD^+ released	1–2	ca. 1
Stability of enzyme-inhibitor complex before borohydride reduction (exposure to 8 M urea or 1% SDS/0.1% TFA at room temp.)	all radioactivity released	all radioactivity released
Stability of enzyme-inhibitor complex after borohydride reduction (exposure to 8 M urea or 1% SDS/0.1% TFA at room temp.)	ca. 0.5–0.6 eq of inhibitor remains	all radioactivity released

in antiviral potency observed for SAH hydrolase inhibitors in different cell cultures could be due at least in part to variations in the cofactor content of the enzyme. This possibility and the reasons for the existence of more than one form of the enzyme should provide interesting subjects for future investigations.

Acknowledgements

We would like to thank Dr. William Plunkett for a generous gift of [2-^{3}H]-deoxy-2',2'-difluoroadenosine. We also are pleased to acknowledge financial support of these investigations by The National Institutes of Health (GM26166) and the Robert A. Welch Foundation (C-729).

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