J. Enzyme Inhibition, 1990, Vol. 4, pp. 1–13
Reprints available directly from the publisher
Photocopying permitted by license only

THE MECHANISM OF INHIBITION OF S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE BY FLUORINE-CONTAINING ADENOSINE ANALOGS

SHUJAATH MEHDI*, ESA T. JARVI, JACK R. KOEHL, JAMES R. McCARTHY and PHILIPPE BEY

Merrell Dow Research Institute, 2110 E. Galbraith Road, Cincinnati, Ohio 45215, USA

(Received 5 September 1989)

(Z)-4′,5′-Didehydro-5′-deoxy-5′-fluoroadenosine (**I**), 5′-deoxy-5′-difluoroadenosine (**II**), and 4′,5′-didehydro-5′-deoxy-5′-fluoroarabinosyl-adenosine (**III**) are inhibitors of rat liver S-adenosyl-L-homocysteine hydrolase. Compounds **I** and **II** are time-dependent and irreversible inhibitors of the enzyme. Both **I** and **II** are oxidized by E.NAD to produce E.NADH, and fluoride anion is formed in the inactivation reaction (0.7 to 1.0 mole fluoride/mole of enzyme subunit, and 1.7 moles fluoride/mole of enzyme subunit from **I** and **II**, respectively). The enzyme is stoichiometrically labeled with [8-^{3}H]-I, but the label is lost upon denaturation of the protein either with or without treatment of the labeled complex with sodium borohydride. The compound **III**, the arabino derivative of **I**, is a competitive inhibitor of the enzyme. The mechanism of the inhibition of S-adenosyl-L-homocysteine hydrolase by these inhibitors is discussed.

KEY WORDS: S-Adenosyl-L-homocysteine hydrolase, time-dependent inhibition, fluorine-containing adenosine analogs, inactivation mechanism.

INTRODUCTION

In many biological methylations, the donor of the methyl group is S-adenosylmethionine.[1] The products of the reaction are the methylated acceptor and S-adenosylhomocysteine (SAH). *In vivo*, the concentration of SAH, which is an inhibitor of several methyltransferases,[2] is presumed to be controlled by the action of the enzyme S-adenosyl-L-homocysteine hydrolase (SAHase; EC 3.3.1.1.), which catalyzes the reversible cleavage of SAH into homocysteine and adenosine.[3] The equilibrium of the reaction favors synthesis but the reaction may be driven in the cleavage direction by the efficient removal of adenosine and homocysteine through other enzymatic reactions.[1] It has been proposed that inhibitors of SAHase may indirectly block methylation reactions by causing an increase in the intracellular concentration of SAH,[2,4] provided that no other means of degradation of SAH are available and its efflux is negligible. Indeed, an increase in intracellular concentration of SAH by SAHase inhibitors has been demonstrated.[5–8] Inhibitors of SAHase have antiviral properties, possibly due to the suppression of viral mRNA methylation necessary for viral replication.[9–11]

The mechanism by which SAHase catalyzes the cleavage of a thioether bond (or in the opposite direction, the formation of a thioether bond) has been delineated by

*Correspondence.

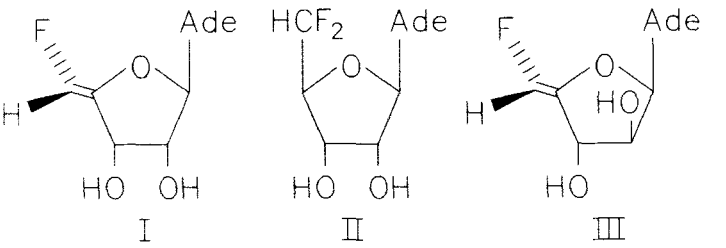


FIGURE 1 Structures of compounds used in this study (Ade = adenine).

Palmer and Abeles.[12,13] The enzyme contains 1 mole of tightly bound β-nicotinamide adenine dinucleotide (NAD) per mole of enzyme subunit[12–14] which is transiently reduced to its reduced form, NADH, and then oxidized back to NAD during one catalytic cycle, with concomitant oxidation and then reduction of the substrate. A few other enzymes utilize a similar mechanistic stratagem to bring about a transformation that would otherwise be difficult[15]. In the reaction catalyzed by SAHase, the first step in the cleavage direction is the oxidation of the 3′-OH of the substrate SAH to the 3′-ketone by E.NAD. This facilitates the abstraction of the 4′-H and the elimination of homocysteine. Water then adds to the enone formed in the elimination, and the product is reduced to adenosine by the E.NADH formed in the first step. On the basis of this mechanism, the compounds **I**, and **II**, and **III** (see Figure 1) were synthesized as inhibitors of SAHase. We report here a study of the mechanism of the inhibition of the enzyme by these compounds. A preliminary account of some of the experiments with **I** has been published in a communication.[11]

MATERIALS AND METHODS

Common biochemicals, S-adenosyl-L-homocysteine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and calf spleen adenosine deaminase were from Sigma. Neplanocin A was a gift from Toyo Jozo Co., Japan. The synthesis and characterization of (Z)-4′,5′-didehydro-5′-deoxy-5′-fluoroadenosine (**I**), 5′-deoxy-5′-difluoroadenosine (**II**), and 4′,5′-didehydro-5′-fluoro-arabinosyl-adenosine (**III**) (Jarvi *et al.*, manuscript in preparation) will be reported elsewhere. Radiolabeled **I** was prepared from unlabeled material by exchange of the hydrogen at the 8 position of the adenine with ^3H from ^3H_2O under basic conditions. In ^2H_2O under the reaction conditions (1 M O^2H^-, room temperature), selective exchange of the 8-H by ^2H was demonstrated by ^1H NMR (Donald P. Matthews and James R. McCarthy, unpublished observations). The exchange reaction was carried out by Amersham, and the labeled material was purified by Dr. Eugene R. Wagner of the Radiochemistry Laboratory at the Merrell Dow Research Institute in Indianapolis, Indiana. Protein was determined by the method of Schaffner and Weissmann[16] and a molecular weight of 48 kD for the enzyme subunit was used to calculate the molarity of enzyme solutions. Stock solutions of inhibitors were made in the assay buffer or in dimethyl sulfoxide (DMSO). The concentration of inhibitor in aqueous solution was determined from the absorbance at 260 nm ($\varepsilon = 15.4\,mM^{-1}\,cm^{-1}$). A Hewlett Packard HP8452 instrument was used for spectrophotometric enzyme assays and UV/visible spectral measurements. Gel filtration (desalting) was performed using a Pharmacia FPLC system and a

Pharmacia fast desalting column (containing Sephadex G-25 superfine) eluted with 1 mM ethylenediaminetetraacetic acid (EDTA)/10 mM potassium phosphate (pH 7.5) at 2 ml/min. Under these conditions, protein eluted between 2 min and 4 min. A Beckman LS 330 instrument was used to measure radioactivity by liquid scintillation counting. ^{19}F Nuclear magnetic resonance (NMR) spectra were obtained using a Varian VXR 300 spectrometer (282 MHz for ^{19}F). Chemical shifts reported are relative to external fluorotrichloromethane.

Preparation and Assay of Rat Liver SAHase

SAHase from rat liver was purified through the first chromatographic step (anion-exchange chromatography using Whatman DE 52) as described by Fujioka and Takata[17] except that phenylmethylsulfonyl fluoride (1 mM) was added to the crude homogenate. Fractions from the anion-exchange column containing enzyme activity were pooled and the protein was precipitated by addition of ammonium sulfate (70 g/100 ml). The protein was pelleted by centrifugation of the suspension at 20,000 g for 20 min. The pellet was dissolved in 50 ml of 10 mM potassium phosphate (pH 6.8) and residual ammonium sulfate was removed by repeated concentration of the solution (Amicon ultrafilitration cell, PM 10 membrane) and addition of buffer. The solution was applied to a column (2.6 cm × 22 cm) of hydroxylapatite (Bio Gel HTP, Bio Rad) previously equilibrated with 10 mM potassium phosphate (pH 6.8). The column was washed with 300 ml of the equilibration buffer and then eluted with a linear gradient (1 L + 1 L) of 10 mM potassium phosphate (pH 6.8) to 100 mM potassium phosphate (pH 6.8). Fractions containing enzyme activity were pooled and concentrated using an Amicon cell with a PM 10 membrane and the buffer was exchanged with storage buffer, composed of 8 mM potassium phosphate (pH 7.2), 5 mM dithiothreitol (DTT), 2 mM EDTA and 20% v/v glycerol. The enzyme was stored at −20°C until use and the activity was stable for at least 6 months. The enzyme (approx. 10 mg) was > 90% pure as judged by FPLC and gel electrophoresis, and the specific activity of the enzyme, assayed with SAH as the substrate as described below, was 1.2 μmole/min/mg (K_m for SAH = 9.6 μM). The enzyme used in some experiments (three of the four ^{19}F NMR experiments) had a specific activity of 0.86 μmole/min/mg. The specific activity reported for the rat liver enzyme is 1.1 μmole/min/mg.[17] SAHase activity was measured spectrophotometrically[7] at 25°C by following the formation of homocysteine, which was detected by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to give the chromophoric thiolate ($\varepsilon_{412\,nm}$ = 13.6 $mM^{-1}\,cm^{-1}$). The reaction mixture (3.0 ml) had the following composition: 100 μM DTNB, 62.5 μM S-adenosyl-L-homocysteine, 62.5 μM S-adenosyl-L-homocysteine, 0.6 U adenosine deaminase (bovine spleen), and SAHase in 1 mM EDTA/10 mM potassium phosphate (pH 7.5). Enzyme stock solutions containing DTT were desalted by FPLC before assay of enzyme activity.

Determination of Inhibition Constants

Initial rates from experiments in which both the inhibitor and the substrate concentrations were varied were used to determine the K_i and the type of inhibition for the reversible inhibitor **III**. The data were analyzed by the Marquardt method and by double reciprocal plots using the kinetics program of the HP8452 spectrophotometer.

The error was within 10%. For the time-dependent inhibitors **I** and **II**, the inactivation reaction was monitored continuously in the presence of substrate and inhibitor.[18] The progress curves were fitted to the equation for a first order process using the fitting program of the kinetics software of the spectrophotometer and the pseudo-first-order rate constants k_{obs} at different inhibitor concentrations were determined. K_I and k_{inact} were obtained from a plot of $1/k_{obs}$ versus $1/[I]$, where slope $= \{K_I(1 + [S]/K_m)\}/k_{inact}$ and y-intercept $= 1/k_{inact}$. The k_{inact} and K_I for **II** were also determined by the method of Kitz and Wilson[19] in which the enzyme and inhibitor were incubated together in the absence of substrate and a portion was removed at intervals to determine enzyme activity. To test for reversibility of inhibition by **I**, **II** or by neplanocin A (a naturally-occurring irreversible inhibitor of SAHase),[7,20] a sample of the inactivated enzyme was gel filtered to remove excess inhibitor and the protein fraction was assayed for enzyme activity. In addition, enzyme inactivated by **I** or neplanocin A was gel filtered and the protein was incubated with NAD (0.5 mM) or without NAD in the gel filtration buffer for 90 min at 37°C and assayed for return of enzyme activity. A sample of active enzyme was taken through the same steps to correct for any effect on the activity.

Determination of NADH Formation

To observe the formation of reduced cofactor, a tandem mixing cell (Hellma; total path length 0.88 cm) was used. The thermostatted cell (25°C) contained enzyme in 0.6 mL (3.6 μM subunits in 1 mM EDTA/10 mM potassium phosphate, pH 7.5, also containing 1.67 mM DTT and 6.7% glycerol from the enzyme storage buffer) in one compartment, and the inhibitor in 0.6 mL (11.5 μM **I**, 430 μM **II** or 330 μM **III**) in the other compartment. After recording the spectrum against air, the spectrophotometer was referenced against the unmixed contents (the absolute absorbance at 340 nm versus air was not greater than 0.07 AU). The contents were then mixed and the spectrum obtained periodically. The final concentrations of enzyme and inhibitor were half the concentrations given above for each compartment. The first-order rate constant for the increase in absorbance at 340 nm at the particular inhibitor concentration used was calculated by fitting the data (absorbance at 340 nm at different times after mixing) to the equation for a first-order process using an Hewlett Packard HP85 computer and the Data Evaluation Program from R & L Software (Newton, MA). In one experiment, the spectral measurements were made in the absence of DTT or glycerol. Before use, the enzyme was transferred to 1 mM EDTA/10 mM potassium phosphate (pH 7.5) from storage buffer by repeated concentration using an Amicon Centricon-30 microconcentrator unit (containing a YM 30 membrane) and addition of the new buffer.

To determine if the NADH formed by the reaction of **I** and E.NAD is enzyme bound, a sample of enzyme inactivated by **I** was gel filtered using Sephadex G-50-80 (1 cm × 12 cm column). The protein peak was collected and its UV/visible spectrum was measured and compared with the spectrum of native enzyme. The fractions that contained any dissociated NADH were also collected and the fluorescence spectrum was obtained (excitation at 340 nm, emission 400 to 500 nm) using an SLM Aminco SPF-500C instrument to detect NADH.

^{19}F NMR Spectroscopy to Detect Fluoride Anion Formation

In 0.82 ml of 1 mM EDTA/10 mM potassium phosphate (pH 7.5), 0.46 mg (9.8 nmole of enzyme subunits) of SAHase (specific activity 1.2 μmole/min/mg) was incubated with 75 nmole of **I**. After 10 min at room temperature, < 2% of the original enzyme activity could be detected. Ethanol (2 ml) was added, the mixture was kept at 0°C for 5 min and then centrifuged. The supernatant was removed and the pellet was washed once with H_2O/EtOH and the combined supernatant and washing was taken to dryness by rotary evaporation followed by lyophilization. The residue was dissolved in ^2H_2O and some insoluble denatured protein present was removed by centrifugation. The ^{19}F NMR spectrum of the sample was obtained. In two experiments in which enzyme of lower specific activity (0.86 μmole/min/mg) was used, the protein was not denatured after the incubation with **I** and was removed instead by filtration of the reaction mixture through a Centricon-30 microconcentrator. In one of these experiments, the concentration of active enzyme subunits in the incubation was 27.5 μM (corrected for lower specific activity and assuming a maximum specific activity of 1.2 μmole/min/mg), and the concentration of **I** was 69 μM; in the second experiment, the active enzyme subunits and **I** were present at 21.2 μM and 69 μM, respectively. After incubation at room temperature for 15 min, less than 2% of the original activity could be detected. The reaction mixture was filtered through a Centricon-30 filter and the residue was washed three times with water. The combined filtrate and washings was lyophilized and the residue was dissolved in ^2H_2O for analysis by ^{19}F NMR spectroscopy. In the experiment with the difluoromethyl compound **II**, enzyme (16.2 μmole of active subunits) and 216 μmole of **II** were incubated for 2.5 h at room temperature. The enzyme had lost 92% of the original activity. The reaction mixture was prepared for ^{19}F NMR spectroscopy by Centricon-30 filtration, washing, and lyophilization as above.

In these experiments, the moles of fluoride anion formed in the reaction was calculated from the NMR spectrum as follows: moles of F^- = [(integral of fluoride signal)/(total integral of inhibitor and fluoride signal)] x moles of inhibitor used in the incubation. In the case of the inhibitor **II** which contains two fluorine atoms/mole, the integral for the NMR signal of the unreacted inhibitor was divided by 2.

To measure the relative recovery of **I** and fluoride, a mixture containing about 5 mM of each was passed through a Centricon-30 microconcentrator and the filter was washed three times with water. A sample of the unfiltered mixture and the combined filtrate and washings were lyophilized and the residues were dissolved in ^2H_2O for analysis by ^{19}F NMR spectroscopy. Ratios of F^- to **I** obtained from spectra acquired with and without a 22 s pulse delay were within 10% (measured T_1 = 2.3 s for F^-, 1.0 s for the fluorine in **I**).

*Labeling Studies with [8-^3H]-**I***

Enzyme from the stock solution (1 nmole subunits) was incubated with 6.3 nmole 8-^{3}H-**I** (specific radioactivity = 93.6 μCi/μmole) in 0.63 ml of 1 mM EDTA/10 mM potassium phosphate (pH 7.5) for 15 min at room temperature. The sample was gel filtered using a Pharmacia fast desalting column. The protein peak was collected and a portion was used to determine protein concentration and another portion to determine ^3H. Portions of the labeled protein (containing approximately 2.4 μg of protein) were also treated to denature the protein (heat at 100°C for 2 min, addition

of 10 vols. of ethanol, or addition of 2 vols. of 10% trichloroacetic acid), centrifuged, and radioactivity in the pellet and supernatant was measured. A sample of heat-denatured labeled protein was also filtered through an Amicon Centricon-30 centrifugal concentrator and the radioactivity in the retentate and the combined filtrate and washings was determined. In another set of experiments, 0.05 nmole of ^3H labeled enzyme (isolated as described above) in 0.6 ml of 0.4 M potassium phosphate (pH 8.0) was treated over 10 min with three 100 μl portions of 100 mM sodium borohydride ($NaBH_4$; freshly dissolved for each addition in 0.5 M potassium phosphate, pH 8.0). The solution was then heated at 100°C for 5 min and filtered through a Centricon-30 concentrator. The filter was washed once, and the radioactivity in the retentate and filtrate/washings was determined. In another experiment, the labeled protein in 0.6 ml of 0.5 M potassium phosphate (pH 8.0) was treated with two 100 μl portions of 100 mM $NaBH_4$ in H_2O. One portion of the $NaBH_4$-treated sample was heated at 100°C for 3 min, a second portion was treated with excess ethanol, and a third portion was untreated. Each sample was filtered through a Centricon-30, the filter was washed, and the radioactivity in the retentate and the filtrate/washings was determined. In experiments using the Centricon microconcentrator, > 90% of the total radioactivity applied was recovered in retentate and filtrate. In one experiment, the enzyme was transferred from storage buffer containing DTT and glycerol to 1 mM EDTA/10 mM potassium phosphate (pH 7.5) using a Centricon-30 filter as described above. After labeling and gel filtration, one portion of the labeled enzyme was heated at 100°C for 2 min to denature the protein and the sample was filtered through a Centricon-30 unit. Another portion was treated with borohydride, the protein was denatured by heat, and the sample was filtered through a Centricon-30 unit. Radioactivity was measured in the residue and in the combined filtrate and washings from the two experiments.

RESULTS

Inhibition Constants

The K_I and k_{inact} for the time-dependent inhibitors **I** and **II**, and the K_i for the reversible inhibitor **III** are presented in Table I. The vinyl fluoride **I** is a 4-fold poorer inhibitor of the rat liver enzyme than of the mouse liver enzyme[11] by comparison of k_{inact}/K_I. The inhibition by either **I**, **II** or neplanocin A was not reversed by gel

TABLE I
Inhibition Constants for SAHase inhibitors

Compound	Type of Inhibition	K_I (μM)	k_{inact} (min^{-1})	K_i (μM)
I	irreversible, time-dependent	6.5	0.8	–
II	irreversible, time-dependent	9.7 (42)[a]	0.04 (0.03)[a]	–
III	reversible, competitive	–	–	11.0

[a]Values determined by the method of Kitz and Wilson[19].

filtration. Inhibition of SAHase by **I** or neplanocin A was not reversed (< 5% return of activity) by incubation of the inactivated enzyme in 1 mM EDTA/10 mM potassium phosphate buffer (pH 7.5) with or without NAD at 37°C for 90 min. A control sample of enzyme incubated under the same conditions lost approximately 10% of the activity. The compound **III** was found to be a competitive inhibitor.

NADH Formation

A time-dependent increase in absorbance at 340 nm was observed when **I** or **II** was mixed with enzyme (Figure 2), which was presumed to be due to the formation of NADH. The total absorbance change corresponds to 1.5 times the concentration of enzyme subunits present, assuming that the extinction coefficient of the NADH is $6.2\,mM^{-1}\,cm^{-1}$. This stoichiometry may be due to a higher extinction coefficient of the enzyme-bound NADH. If more inhibitor was added after the initial increase, no further change in absorbance was observed. No significant difference was observed in either the rate or the extent of NADH formation in the absence of DTT in the reaction mixture. To determine if the NADH is free or enzyme bound, enzyme inactivated by **I** was separated from excess inhibitor and any dissociated cofactor by gel filtration. Comparison of the UV/visible spectrum of the inactivated and gel filtered protein with the spectrum of native enzyme showed that NADH is enzyme-bound. No NADH was detected (< 20% of maximum) by fluorescence in the fractions in which NADH would be expected to be present. The rate of increase in absorbance at 340 nm was determined from a plot of absorbance at 340 nm versus time (Figure 3). A first-order rate constant of $0.45\,min^{-1}$ characterizes the rate of increase in absorbance at 340 nm at the inhibitor concentration of 5.8 μM. If it is assumed that this process is described by the same K_I as the inactivation reaction, then the pseudo-first-order rate constant at saturating inhibitor concentration (analogous to k_{inact}) for the absorbance increase is $1.0\,min^{-1}$. Similarly, the pseudo-first-order rate constant for the absorbance increase at saturating **II** is $0.02\,min^{-1}$. No change in absorbance (< 10% of the maxim-

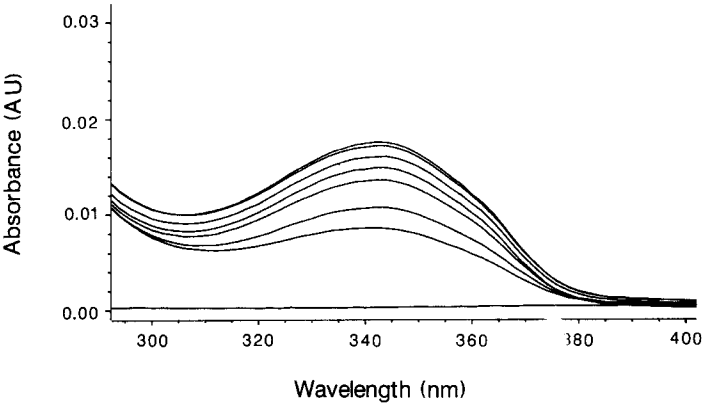


FIGURE 2 Spectra of a mixture of SAHase and **I** in a tandem divided cell obtained before mixing and 1.25, 2, 4, 6, 8, 10, and 14 min after mixing (in order of increasing absorbance at 340 nm). Conditions were as described in the experimental section. The instrument was referenced against the unmixed contents of the cell; before mixing, the absolute absorbance at 340 nm (versus air) was 0.055 AU.

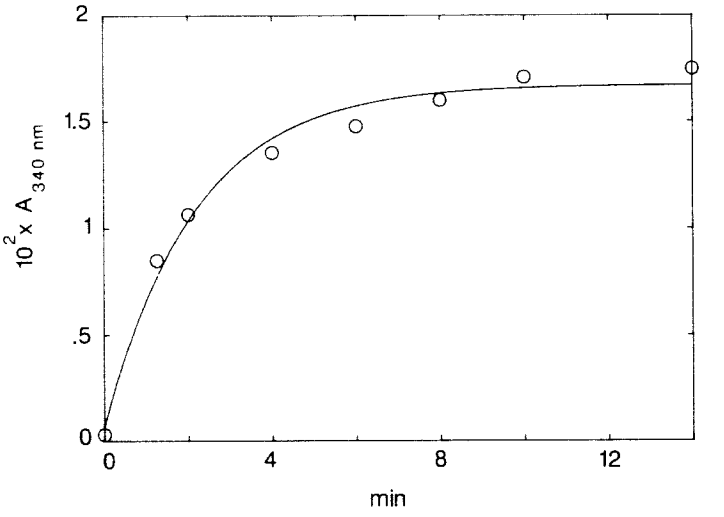


FIGURE 3 A plot of absorbance at 340 nm versus time from the experiment described in Figure 2. The curve is the fit of the data to the equation for a first-order process.

um) was seen for up to 60 min when the enzyme was mixed with the reversible inhibitor **III** at a concentration 15 times its K_i.

Release of Fluoride Anion

The release of fluoride in the reaction of **I** or **II** with the enzyme was measured by ^{19}F NMR spectroscopy. In three experiments with the inhibitor **I**, it was found that 1.03, 0.78, and 0.65 mole of fluoride was formed per mole of enzyme subunits in the reaction mixture from which the NMR sample was derived. The ^{19}F NMR spectrum (^1H coupled) obtained in the first experiment is shown in Figure 4. The signal due to fluoride anion is at -120.7 ppm (no coupling to ^1H); this signal increased when sodium fluoride was added to the NMR sample. The doublet at -162.5 ppm is due to unreacted **I** ($J_{F\text{-}C\text{-}H} = 74.2$ Hz). No other signals were present. In this experiment, inactivated enzyme was removed from the reaction mixture after incubation with the inhibitor by denaturation with ethanol and centrifugation. The supernatant was concentrated and analyzed by NMR spectroscopy. In the two experiments in which less than stoichiometric amounts of fluoride were found, the enzyme was removed after the incubation by filtration through a Centricon-30 microconcentrator followed by washing with water. The ^{19}F NMR spectrum of the combined filtrate and washings was obtained after evaporation. In these two experiments, it was assumed that the actual concentration of the enzyme subunits was 75% of that found by protein assay, since the enzyme had a specific activity of 0.86 μmole/min/mg, compared with 1.2 μmole/min/mg of fully active enzyme.

Filtration through a Centricon-30 unit of a mixture of fluoride and **I** followed by washing with water did not give selective enrichment of either component, i.e. the relative recovery was the same (within 1%).

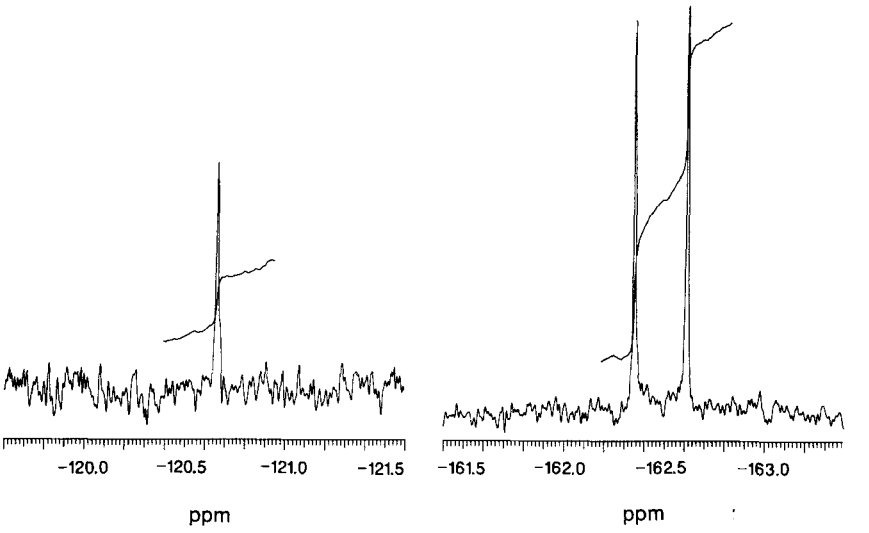


FIGURE 4 The ^{19}F NMR spectrum of the supernatant from the reaction of SAHase and **I** as described in the experimental section. A total of 16,5553 transients were collected (acquisition time = 0.3 s, no pulse delay) using a spectral width of 23923.4 Hz. Chemical shifts are relative to external fluorotrichloromethane.

In the experiment in which the inhibitor **II** was reacted with SAHase, the enzyme was removed from the reaction mixture by microfiltration. NMR analysis of the supernatant after work-up showed signals due to fluoride anion (−122.3 ppm) and unreacted **II** (doublet of doublets centred at −130.21 ppm; J_{F-C-H} 54.1 Hz and $J_{F-C-C-H}$ = 13.3 Hz). The ratio of moles of fluoride released to moles of enzyme subunits was 1.66 (this number is corrected for both the extent of inactivation, 92%, in the experiment and the lower specific activity of the enzyme used in the experiment).

*Labeling of SAHase with [8-^{3}H]-**I***

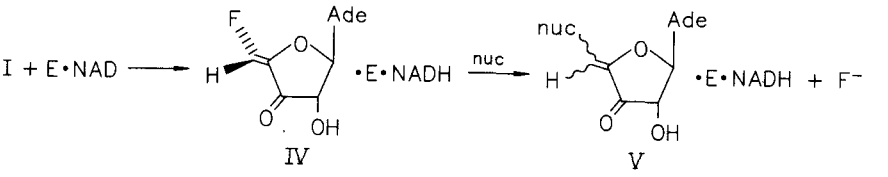
SAHase is labeled stoichiometrically with [8-^{3}H]-**I**; in two experiments, one mole of enzyme subunit was found to bind 0.88 and 0.77 mole of the inhibitor. The label was not removed by rapid gel filtration, but >93% of the label was lost under three different denaturing conditions. Treatment of the labeled enzyme with sodium borohydride failed to prevent the release of label upon denaturation of the protein. In a series of experiments, 83 ± 1% of the label was lost from borohydride-treated and denatured complex, as determined by microfiltration, compared with 80 ± 5% from control samples (denatured but not treated with reducing agent). If the complex was treated with borohydride but not denatured, only 30% of the label was lost. The presence of DTT did not effect the results; in one experiment in which the reaction was free of DTT (from the enzyme storage buffer), 95% of the label was lost by heat denaturation, from either borohydride treated or untreated samples.

DISCUSSION

Irreversible inhibition of rat liver SAHase by the inhibitors **I** and **II** is accompanied by the formation of enzyme-bound NADH. By analogy with the oxidation of the substrate and the oxidation of other irreversible inhibitors,[21–23] it is most likely that the site of oxidation of **I** or **II** is the 3′ position. The competitive inhibitor **III**, which differs from **I** only in that it has the opposite configuration at the 2′ position, is not oxidized; thus, at least one step of the normal reaction pathway – the oxidation at 3′ – is necessary for time-dependent inhibition of SAHase. Inhibitors **I** and **II** can therefore be classified as mechanism-based inhibitors.[24] It is interesting that **III** is not oxidized by SAHase, whereas the 2′ epimer of adenosine (9-β-D-arabinofuranosyl-adenine, or ara-A) is oxidized by the enzyme.[5]

In the case of the vinyl fluoride **I**, the first-order rate constant for the formation of enzyme-bound NADH ($1.0\,min^{-1}$) is similar to the first-order rate constant for inactivation of the enzyme ($0.8\,min^{-1}$), while the corresponding rate constants for **II** ($0.02\,min^{-1}$ for NADH formation and $0.04\,min^{-1}$ for inactivation) are somewhat different. A possible explanation for the difference in these rates for **II** is that the rate constant for inactivation, measured by following the inactivation reaction continuously in the presence of inhibitor and substrate, reflects not only the inactivation by **II** but also a slow inactivation of the enzyme by DTNB (the active site is known to contain a sulfhydryl group essential for activity[25]). Thus, a smaller value for k_{inact} and a higher K_I was obtained for the inhibitor **II** using the more traditional Kitz and Wilson method (see Table I). The rate of reaction with DTNB is slow enough not to significantly affect the measurement of initial rates or the rate of inactivation by an efficient time-dependent inhibitor such as **I** which does not require a lengthy assay.[26] Determination of the inhibition constants of **I** by the continuous assay proved convenient, since these values could not be easily determined by the Kitz and Wilson method (because of the low sensitivity of the assay, the inherently low specific activity of the enzyme, and the low K_I of the inhibitor, the enzyme concentration would approach the inhibitor concentration in the incubation mixture). The inhibition of SAHase by **I** was not reversed by incubating the inactivated enzyme with NAD, although reactivation of neplanocin-inactivated bovine SAHase has been reported.[27]

The availability of relatively large amounts of enzyme permitted the determination of fluoride anion formation directly by ^{19}F NMR spectroscopy. After incubating with inhibitor and ensuring complete or nearly complete inactivation, the enzyme was removed either with or without denaturation. Thus, the fluoride ion observed in the NMR spectrum must be produced in the initial inactivation reaction, and not by the subsequent breakdown of a fluorine-containing enzyme-bound species either during the denaturation reaction or in a slow reaction during acquisition of the NMR spectrum. In the experiments with the inhibitor **I**, 1 mole of fluoride/mole of enzyme subunit was found in one experiment but in two other experiments, somewhat less than stoichiometric amounts were detected. In the experiment with the inhibitor **II**, 1.66 mole fluoride/mole of enzyme subunits was found, twice the amount detected in the two experiments with the inhibitor **I**, suggesting that 2 moles of fluoride are formed per mole of enzyme subunits.[28] If it is reasonably assumed that the fluoride anion and unreacted inhibitor observed in the ^{19}F NMR spectra account for all the fluorine-containing species, then the stoichiometric fluoride release shows that the partition ratios (the ratio of turnover events to inactivation events) for both **I** and **II**



are close to zero; i.e., every catalytic event leads to inactivation. This is true regardless of the mechanism of the inhibition, since it is based only on the molar equivalence.

Labeling of the enzyme with tritium-labeled **I** was attempted. Although the enzyme was stoichiometrically labeled and the label was not removed by rapid gel filtration, denaturation even under mild conditions caused the label to dissociate from the protein. Treatment of the labeled protein isolated by gel filtration with a large excess of sodium borohydride failed to prevent the release of the label upon denaturation by heat or with ethanol.

Scheme 1 (above) shows the minimal mechanism for the inhibition of SAHase by **I** that is supported by our data. Oxidation of the inhibitor by E.NAD gives the enone **IV**, and the NADH formed stays enzyme-bound (it has been shown that the cofactor in either its reduced or its oxidized form can dissociate from the enzyme under certain conditions[29]). The enone **IV** undergoes nucleophilic attack followed by fluoride elimination[30] to give **V**. Since inhibition of SAHase by **II** is accompanied by the generation of two moles of fluoride per mole of inactivated enzyme subunit, it is reasonable to assume that a vinyl fluoride is generated as an intermediate in the reaction by abstraction of the 4′ hydrogen and elimination of one mole of fluoride anion. The reaction would then proceed as depicted for **I** in the Scheme, resulting in the release of a second mole of fluoride.

While there is good evidence for the overall pathway shown in the Scheme, the identity of the attacking nucleophile and the nature of the inactive enzyme-inhibitor complex are less clear. If the nucleophile is either an –SH or an –OH group of the enzyme, the resulting adduct **V** is expected to be relatively stable and therefore the label should not be lost from the denatured enzyme. If the attacking nucleophile were a lysine amino group, the resulting adduct might be more labile with respect to hydrolysis if it tautomerizes to a Schiff base (however, this will remove the double bond from conjugation with the carbonyl). It is expected that reduction by borohydride of the Schiff base will stabilize the linkage towards hydrolysis; reduction in either the 1,2- or 1,4-mode should also stabilize further an adduct with either a thiol or hydroxyl nucleophile.[31] If the attacking nucleophile were the carboxylate group of a glutamate or an aspartate residue, an enol ester would be formed which might hydrolyze even under mild conditions; this lability would not be affected by reduction. We observe loss of label from the protein under mild denaturing conditions with or without borohydride treatment, which is consistent with at least one of the following: (1) the attacking nucleophile as shown in the Scheme is a water molecule and not a nucleophile of the enzyme (see below); (2) the reducible group of the bound inhibitor is not accessible to the reducing agent (however, Palmer and Abeles[12,13] successfully trapped the 3′-keto form of the adenosine analog 5′-deoxyadenosine); and (3) the attacking nucloeophile is a carboxylate group of the enzyme. The finding that the partition ratio for **I** and **II** is zero shows that every step in the inactivation by either compound must proceed with 100% efficiency. In a mechanism in which trapping by

an enzyme nucleophile is a necessary step, inhibition by **I** requires at least two steps and inactivation by **II** requires at least three steps. If this were indeed the mechanism by which SAHase is inactivated by the inhibitors **I** and **II**, the oxidized inhibitor formed initially must dissociate slowly enough for the subsequent steps to occur with complete efficiency.

In the normal reaction, the enzyme catalyzes the addition of water to the enone derived from adenosine. Thus, in one possible mechanism for the inactivation of the enzyme by **I** and **II**, the attacking nucleophile in the Scheme could be a water molecule rather than a nucleophile of the enzyme. If this were the case, the species **V** or one of its tautomeric or hydrated forms may be the tightly bound species, and thus addition of water would be necessary for irreversible inactivation. Alternatively, it may be necessary only to tie up the enzyme in the oxidized form to achieve inactivation, and the release of fluoride by attack of water (or even by a nucleophile of the enzyme) may be adventitious. Abeles and co-workers[21] have suggested that reduction of SAHase.NAD to SAHase.NAD converts the enzyme to a "closed" form that does not release bound molecules, and a mechanism involving simply the conversion of SAHase to the inactive E.NADH form accompanied by tight binding of the oxidized inhibitor has been proposed by Borchardt's group for the inhibition of SAHase by neplanocin A and its analogs[22,23] Thus, it may be that the irreversibility of the inhibition of SAHase by at least some inhibitors may be due to the tight noncovalent binding of the oxidized inhibitor to the reduced enzyme rather than through covalent attachment. An example of an inhibitor whose oxidized form appears to be tighly, but noncovalently, bound to the reduced form of an enzyme is allopurinol. The inhibitor is oxidized by xanthine oxidase to alloxanthine, which remains tighly bound to reduced xanthine oxidase.[32] Although the mechanism of inactivation would be different, there need not be any difference from a practical viewpoint between inactivated enzyme arising from covalent attachment of an inhibitor and enzyme inactivated by tight binding of a form of an inhibitor.

Acknowledgements

We are grateful to Dr. Ronald T. Borchardt for helpful discussions and for giving us a manuscript prior to its publication. We thank Dr. Eugene Wagner for the purification of radiolabeled material. We are grateful to Dr. Ed Huber for assistance in obtaining NMR spectra. We also thank Charlene Mercer and Steve Biedenbach for help in preparation of the manuscript.

References

1. Ueland, P.M. (1982) *Pharmacol. Rev.*, **34,** 223.
2. Borchardt, R.T. (1980) *J. Med. Chem.*, **23,** 347.
3. de la Haba, G. and Cantoni, G.L. (1959) *J. Biol. Chem.*, **234,** 603.
4. Chiang, P.K. (1984) *Adv. Exp. Med. Biol.*, **165B,** 199.
5. Hershfield, M.S., Small, W.C., Premakumar, R., Bagnara, A.S. and Fetter, J.E. (1981) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, E., Borchardt, R.T. and Creveling, C.R., Eds.) pp. 657. London: Macmillan.
6. Helland, S. and Ueland, P.M. (1982) *Cancer Res.*, **42,** 1130.
7. Wolfson, G., Chisholm, J., Tashjian, A.H., Jr., Fish, S. and Abeles, R.H. (1986) *J. Biol. Chem.*, **261,** 4492.
8. Hasobe, M., McKee, J.G., Borcherding, D.R., Keller, B.T. and Borchardt, R.T. (1988) *Mol. Pharmacol.*, **33,** 713.
9. De Clercq, E. (1987) *Biochem. Pharmacol.*, **36,** 2567.
10. De Clercq, E. (1987) *Nucleosides and Nucleotides*, **6,** 197.

11. McCarthy, J.R., Jarvi, E.T., Matthews, D.P., Edwards, M.L., Prakash, N.J., Bowlin, T.L., Mehdi, S., Sunkara, P.S. and Bey, P. (1989) *J. Am. Chem. Soc.*, **111,** 1127.
12. Palmer, J.L. and Abeles, R.H. (1976) *J. Biol. Chem.*, **251,** 5817.
13. Palmer, J.L. and Abeles, R.H. (1979) *J. Biol. Chem.*, **254,** 1217.
14. Richards, H.H., Chiang, P.K. and Cantoni, G.L. (1978) *J. Biol. Chem.*, **252,** 4476.
15. Frey, P.A. (1987) in *Pyridine Nucleotide Coenzymes* (Dolphin, D., Poulson, R. and Avramovic, O., Eds.) Vol 2B, pp. 461. New York: Wiley.
16. Schaffner, W. and Weissmann, C. (1973) *Anal. Biochem.*, **56,** 502.
17. Fujioka, M. and Takata, Y. (1981) *J. Biol. Chem.*, **256,** 1631.
18. Tsou, C.L. (1988) *Adv. Enzymol,* **61,** 381.
19. Kitz, R. and Wilson, I.B. (1982) *J. Biol. Chem.*, **237,** 3245.
20. Borchardt, R.T., Keller, B.T. and Patel-Thombre, U. (1984) *J. Biol. Chem.*, **259,** 4353.
21. Abeles, R.H., Fish, S. and Lapinkas, B. (1982) *Biochemistry,* **21,** 5557.
22. Paisley, S.D., Hasobe, M. and Borchardt, R.T. (1989) *Nucleosides and Nucleotides,* **8,** 689.
23. Paisley, S.D., Wolfe, M.S. and Borchardt, R.T. (1989) *J. Med. Chem.*, **32,** 1415.
24. Silverman, R.B. (1988) *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology* Vol. 1, Chapter 1. Florida: CRC Press.
25. Gomi, T., Ogawa, H. and Fujioka, M. (1986) *J. Biol. Chem.*, **261,** 13422.
26. SAHase incubated with 100 μM DTNB alone was 70% inactivated in 30 min. However, the inactivation by DTNB is slower in the presence of 62.5 μM substrate (6 times the K_m); under the normal assay conditions, the rate of SAH cleavage at 30 min is 75% of the initial rate. Part of this decrease may be due to substrate depletion (about 20% depletion of SAH in 30 min in a typical assay).
27. Matusvewska, B. and Borchardt, R.T. (1987) *J. Biol. Chem.*, **262,** 265.
28. A possible explanation for the stoichiometry found in two experiments with **I** and one experiment with **II** is that recovery of fluoride is less efficient than the recovery of the unreacted inhibitor from the Centricon-30 filtration step used in these experiments. This is most likely due to nonspecific binding of fluoride anion to the protein under the conditions of low ionic strength, since in a control in the absence of protein, there was no difference between the recovery of fluoride and **I**.
29. de la Haba, G., Agostini, S., Bozzi, A., Merta, A., Unson, C. and Cantoni, G.L. (1986) *Biochemistry,* **25,** 8337.
30. A mechanism for fluoride anion formation that does not involve nucleophilic attack is as follows: loss of the 2′ proton from the oxidized intermediate **IV** to give an enediol, tautomerization of the enediol to form a 2′-keto-3′,4′-didehydro-5′-deoxy-5′-fluoro intermediate, and finally elimination of fluoride with the generation of a 4′,5′-didehydro-2′,3′-diketone. However, this mechanism invokes an unnecessarily large number of steps. Also, there is no evidence to suggest that there is a base in the active site that is positioned for proton abstraction from the 2′ position: Palmer and Abeles[12,13] have detected SAHase-catalyzed exchange at only the 4′ position in adenosine and 5′-deoxyadenosine.
31. The loss of the ^{3}H-labeled adenine by a 1,2 syn elimination or by a stepwise process would also be prevented by reduction of the carbonyl group at the 3′ position of an intermediate.
32. Massey, V., Komai, H., Palmer, G. and Elion, G.B. (1970) *J. Biol. Chem.*, **245,** 2837.