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THE MECHANISM OF INACTIVATION OF S-ADENOSYLHOMOCYSTEINE HYDROLASE BY FLUORINATED ANALOGS OF 5′-METHYLTHIOADENOSINE

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5′-Deoxy-5′-difluoromethylthioadenosine (DFMTA) **1a** and 5′-deoxy-5′-trifluoromethylthioadenosine (TFMTA) **1b** are inhibitors of beef liver S-adenosyl-L-homocysteine hydrolase. DFMTA and TFMTA are time-dependent and irreversible inhibitors of the enzyme. Both **1a** and **1b** are oxidized by E-NAD$^+$ to produce E-NADH and fluoride anion is formed in the inactivation reaction (2.2 mol of fluoride/mole of enzyme subunit and 3.1 fluoride/mole of enzyme subunit from DFMTA and TFMTA respectively). Using [8-^{3}H]-**1a** or [8-^{3}H]-**1b** no trace of labelled adenosine was detected during the inactivation reaction but adenine was formed. The mechanism of inhibition of S-adenosyl-L-homocysteine hydrolase by these two fluorinated nucleosides is discussed.

Keywords: S-adenosyl-L-homocysteine hydrolase; Time-dependent inhibition; 5′-fluoromethylthioadenosines; Enzyme-activated acylating agents

INTRODUCTION

S-Adenosylhomocysteine (AdoHcy) is the product of all biological methylations in which S-adenosylmethionine (AdoMet) is utilized as a methyl donor. This important metabolite is reversibly hydrolyzed to L-homocysteine and adenosine (Ado) by S-adenosylhomocysteine hydrolase (E.C. 3.3.1.1).[1]

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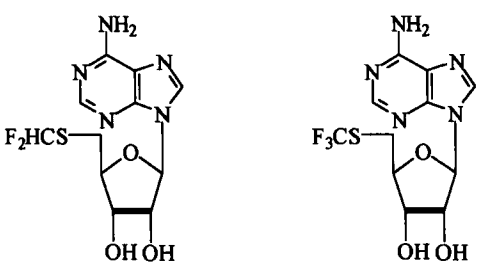
Inhibition of this cellular enzyme results in intracellular accumulation of AdoHcy leading to a feedback inhibition of AdoMet-dependent methylation reactions (i.e. viral mRNA methylations which are essential for viral replication).[2] Therefore, AdoHcy hydrolase has become an attractive target for developing antiviral agents in general and retroviral agents in particular.[3]

During the last decade, the design and synthesis of mechanism-based inhibitors have received considerable attention particularly since the mechanism of the catalysis of AdoHcy hydrolase was elucidated by Palmer and Abeles.[4] In the hydrolytic direction, this mechanism involves oxidation of the 3′-hydroxyl group of AdoHcy by enzyme-bound NAD^+ to give the corresponding 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 reduced to adenosine by enzyme-bound NADH (as shown in Scheme 1, pathway B).

Two classes of potent irreversible inhibitors have been identified for AdoHcy hydrolase. Type I mechanism-based inhibitors are substrates for the C-3′ oxidative action of the enzyme and irreversibly keep the AdoHcy hydrolase in its NADH form, thus disabling the cycle of the overall enzyme reaction.[3b] Type II mechanism-based inhibitors utilize the same oxidative action of the enzyme to generate an electrophilic site on the inhibitor which can then bind with an active site nucleophile.[3c,5]

We report here a novel approach to the irreversible inhibition of AdoHcy hydrolase involving the use of analogs of 5′-deoxymethylthioadenosine **1a** and **1b** (Figure 1) fluorinated at the 5′-methylthio position as enzyme-activated acylating agents. From the study of the differents steps of interaction of **1a** and **1b** with the enzyme the main lines of the mechanism



1a DFMTA **1b** TFMTA

FIGURE 1 Structures of compounds used in this study.

of inhibition with these inhibitors are discussed. A preliminary account of some of the experiments with **1a** has been published in a communication.[6]

MATERIALS AND METHODS

General

Adenosine (Ado), homocysteine (Hcy) were from Aldrich. [8-^{14}C]-Ado was purchased from NEN. ^{19}F NMR spectra (235 MHz, reference $CFCl_3$) were recorded on a Bruker AC 250 spectrometer. Chemical shifts are reported in ppm (δ) and J values in Hz. TLC was performed with precoated silica gel Merck plates. HPLC experiments were carried out on a Shimadzu LC-6A liquid chromatograph equipped with a UV SPD 6AV detector and for radiodetection with a Berthold LB 507-A monitor. A Packard TRI-CARB 1900TR was used to measure radioactivity by liquid scintillation counting.

Protein was determined using bovine serum albumine (BSA) as a standard by the method of Bradford[7]. A molecular weight of 48 kDa for the enzyme subunit was used to calculate the molarity of the enzyme solution. The concentration of inhibitors in aqueous solution was determined from the UV absorbance at 259 nm ($\varepsilon = 13\,265\,M^{-1}\,cm^{-1}$). The inhibition constant and the catalytic turnover number for mechanism inactivation are termed K_i and k_{inact}, respectively.

Chemicals

DFMTA and TFMTA were prepared from adenosine according to the general procedure described by Honek and coworkers[8] via a 5′-deoxy-5′-thioacetate adenosine derivative and fully characterized by ^{1}H and ^{19}F NMR. Since fluorothioethers are susceptible to facile hydrolysis,[9] it was important to determine the stability of these compounds in buffer assay prior to enzymic evaluation. DFMTA and TFMTA proved to be stable in buffer assay over a period of four days (HPLC and ^{19}F NMR controls).

[8-^{3}H]-DFMTA and [8-^{3}H]-TFMTA were prepared from unlabelled material by exchange of hydrogen at the C-8 position of the adenosine moiety with ^3H_2O (Spec. Act. 1.08 mCi/ml) under basic conditions (1 M NaOH).[10] After purification on an hydrophobic HP20SS resin, [8-^{3}H]-DFMTA and [8-^{3}H]-TFMTA were obtained with specific activities of 3.15 and 3.35 μCi/μmol respectively. The chemical homogeneity of these two labelled fluorinated nucleosides was checked by HPLC and TLC, and their radiochemical

purity by radiochromatography using an LB 285 Berthold linear analyzer equipped for CHROMA 2D.

Enzyme Purification

AdoHcy hydrolase was purified to homogeneity from beef livers as described previously[10,11] with a final specific activity of 4 U/mg in the synthetic direction. The degree of purity was assessed by SDS PAGE which showed a simple band corresponding to a MW of 48 000.

Assay of AdoHcy Hydrolase Activity

AdoHcy hydrolase activity was assayed in the synthetic direction by measuring the rate of formation of [8-^{14}C]-AdoHcy from [8-^{14}C]-Ado and Hcy as described previously by Della Ragione *et al.*[12]

Enzyme was mixed with 10 μM [8-^{14}C]-Ado (17 800 dpm) and 5 mM Hcy, 20 mM potassium phosphate buffer pH 7.5, 1 mg/ml BSA, 1 mM EDTA in a final volume of 50 μl. The assay mixture was incubated at 37°C for 10 min and the reaction stopped by addition of 150 μl of 15 mM HCl. One hundred and fifty μl of the sample was then applied to a microcolumn (0.5 ml) of Cellex P equilibrated with 10 mM HCl. The remaining [8-^{14}C]-Ado was eluted first with 5 ml of 10 mM HCl and then the [8-^{14}C]-AdoHcy formed with 5 ml of 0.5 M HCl. The eluates were directly poured into scintillation vials with 7 ml of Ultima Flow AP (Packard) for counting. Under these conditions, the velocity of AdoHcy hydrolase showed normal Michaelis–Menten kinetics with the K_m value for Ado being 3 μM, in accord with the literature value.[13]

One unit of enzyme was defined as the quantity of AdoHcy hydrolase that catalyzes the formation of 1 μmol of AdoHcy per minute under the conditions of the assay described above.

Time-dependent Inactivation Studies

Time-dependent inactivation of AdoHcy hydrolase by **1a** and **1b** was measured by incubating various concentrations of the inhibitor **1a** (2.5–10 μM) or **1b** (50–150 μM) with 6 nM AdoHcy hydrolase in buffer assay at 37°C for different periods of time (0–120 min). The activity remaining was determined in the synthetic direction as described above and kinetic constants calculated according to the method of Kitz and Wilson.[14] Plots of log(percentage of residual activity) *versus* time gave the slopes k_{obs}. Replots of the reciprocal of these slopes *versus* 1/[I] were used to determine the apparent binding constant (K_i) and the apparent inactivation rate constant k_{inact}.

Determination of NADH Formation

The formation of E-NADH during the inhibition process was determined by measuring the increase in absorbance at 320 nm at different time intervals after the enzyme was mixed with inhibitors. AdoHcy hydrolase (7–10 mg) was dissolved in 3.5 ml of 20 mM phosphate potassium buffer pH 7.5. The UV spectrum (280–500 nm) was recorded against 20 mM phosphate potassium buffer pH 7.5 and served as reference (scan A). To the enzyme solution was added the inhibitor solution: 30 µl of **1a** (13 mM) or 70 µl of **1b** (4.5 mM). The UV spectrum (280–500 nm) was recorded periodically against 20 mM phosphate potassium buffer, pH 7.5 (scan B), until enzyme was totally inactivated. Subtraction (B) – (A) was used to measure the increase in absorbance at 320 nm.

Determination of Fluoride Anion Formation

F^- released from DFMTA and TFMTA by incubation with AdoHcy hydrolase was determined by ^{19}F NMR spectroscopy.

In 3.5 ml of 20 mM phosphate potassium buffer pH 7.5, 10.8 mg (1.1×10^{-7} mol of enzyme subunit) of AdoHcy hydrolase (Spec. Act. 2.5 U/mg) was incubated at 37°C with 4×10^{-7} mol of DFMTA. After 92% inactivation of the enzyme, ethanol (9 ml) was added and the mixture centrifuged. The supernatant was removed, the pellet was washed twice with H_2O/ethanol (50/50, 3 ml) and the combined supernatant and washings evaporated to dryness. The residue was dissolved in 0.5 ml of ^2H_2O for ^{19}F NMR analysis. A control experiment conducted under the same conditions without enzyme was made. In the experiment with TFMTA, enzyme (7.3 mg, 1.4×10^{-7} mol of enzyme subunit, Spec. Act. 3.6 U/mg) and TFMTA (7.4 10^{-7} mol) were incubated at 37°C until 85% inactivation of enzyme. The reaction mixture was treated for ^{19}F NMR analysis as described above.

Determination of fluoride anion release from DFMTA and TFMTA in these experiments was calculated from: (^{19}F NMR integral of F^- signal)/(^{19}F NMR total integral of inhibitor and F^- signals) × moles of inhibitor used, taking into account the two or three fluoride atoms/mole present in DFMTA and TFMTA respectively.

Analysis of Reaction Products

AdoHcy hydrolase (22 nmol) was incubated with [8-^{3}H]-DFMTA (66 nmol, 3.15 µCi/µmol) in 500 µl of 20 mM phosphate potassium buffer pH 7.5. After 60% inactivation of the enzyme the reaction was stopped by addition

of 1.5 ml of ethanol. The resulting mixture was centrifuged and the supernatant removed. The pellet was washed twice with $EtOH/H_2O$ and the combined supernatant and washings concentrated by rotary evaporation. The residue was chromatographed on TLC precoated silica gel Merck plates using two sets of eluting solvent, ethyl acetate/MeOH (80/20) and $EtOH/H_2O$ (90/10), and the compounds visualized by UV and radio scanned on a Berthold LB 285 TLC linear analyzer. Unlabelled DFMTA, Ado, and adenine were used as references. In the experiment with TFMTA, AdoHcy hydrolase (23 nmol) was incubated with [8-^{3}H]-TFMTA (93 nmol, 3.35 μC_i/μ mol) in 500 µl of 20 mM phosphate potassium buffer pH 7.5 and the reaction mixture was treated for autoradiography analysis as described above. In a complementary set of experiments the labelled contents of assays were analyzed by HPLC (UV- and radio-detection) on an analytical C18 spherisorb 5 µ (250 × 4.6 cm) column using $MeOH/H_2O$ as eluant, at a flow rate of 0.8 ml/min.

Irreversibility of Inactivation Process

AdoHcy hydrolase (5.3 µg) was incubated at 37°C in assay buffer (1.5 ml) with DFMTA or TFMTA (1 mM). After total inactivation of the enzyme the two assay solutions were dialyzed against 2 l of 20 mM potassium phosphate buffer pH 7.5 for 18 h at 4°C and the activity assayed again and compared with a control containing no inhibitor.

Effect of Adenosine on the Inactivation of AdoHcy Hydrolase by DFMTA or TFMTA

AdoHcy hydrolase (0.1 µg) was preincubated in 50 µl buffer assay for 20 min with the inactivators **1a** or **1b** alone or in the presence of adenosine (14–28 µM). Activity remaining was assayed by transfer of a 10 µl portion of the incubation mixture to 40 µl of standard assay mixture.

RESULTS

Inactivation of AdoHcy Hydrolase by DFMTA and TFMTA

When the purified AdoHcy hydrolase was incubated with DFMTA or with TFMTA a time-dependent loss of activity was observed. The Kitz and Wilson plots of **1a** and **1b** are shown in Figure 2. The kinetic constants thus obtained are listed in Table I.

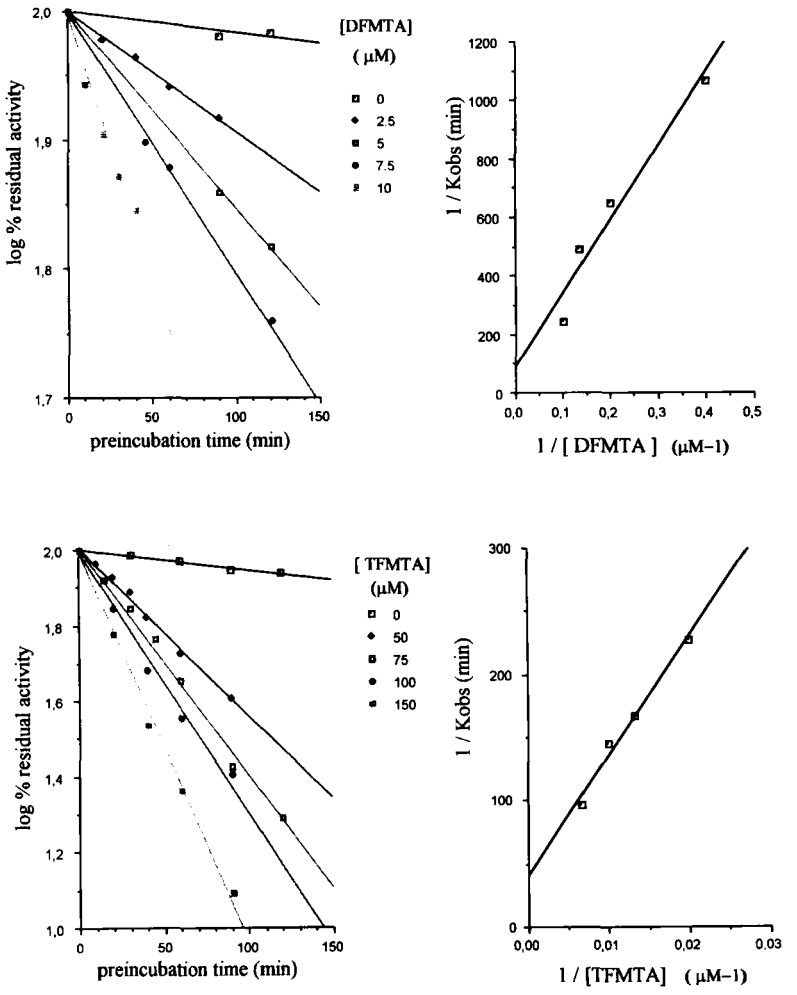


FIGURE 2 Time-dependent inactivation of AdoHcy hydrolase by DFMTA **1a** and TFMTA **1b**. Beef liver AdoHcy hydrolase was incubated with the concentration (μM) shown of DFMTA and TFMTA for the time indicated and the AdoHcy hydrolase activity remaining determined.

TABLE I Inhibition constants for DFMTA and TFMTA with AdoHcy hydrolase

Compound	*Type of inhibition*	K_i (μM)	k_{inact} (min^{-1})	k_{inact}/K_i ($M^{-1}min^{-1}$)	$t^{1/2}$ (min)
DFMTA	Irreversible, time-dependent	28	0.010	357	76[a]
TFMTA	Irreversible, time-dependent	230	0.025	109	71[b]

The half-life ($t_{1/2}$) of enzyme inactivation; (a) at 10 μM of DMFTA; (b) at 50 μM TFMTA.

TABLE II Effect of adenosine on inactivation of AdoHcy hydrolase by DFMTA and TFMTA

Inactivator	*Compound added*	*AdoHcy hydrolase activity remaining* (%)
None	None	100
	Adenosine (28 μM)	82
DFMTA (50 μM)	None	0
	Adenosine (28 μM)	55
TFMTA (50 μM)	None	49
	Adenosine (14 μM)	95
	(28 μM)	82

AdoHcy hydrolase was preincubated for 20 min with inactivators alone or in presence of adenosine. Activity remaining was then assayed.

The half-life ($t_{1/2}$) of DFMTA induced loss of activity at 10 μM was 76 min, TFMTA gave a half-life of 71 min at 50 μM. This loss of activity appeared to be irreversible in that extensive dialysis to remove excess of the inhibitors did not restore activity. The enzyme could be protected against inactivation by DFMTA or TFMTA by the presence of adenosine (Table II).

When buffered AdoHcy hydrolase was mixed with **1a** or **1b**, an increase in the UV absorbance profile at 320 nm was observed which is consistent with formation of enzyme bound NADH (UV spectrum not shown).

Determination of Released Fluoride

Inactivation of AdoHcy hydrolase by DFMTA and TFMTA was accompanied by release of fluoride anion as measured by ^{19}F NMR spectroscopy. As shown in Figure 3, when a 3-fold excess of DFMTA per enzyme subunit was incubated with purified AdoHcy hydrolase, unreacted DFMTA (^{19}F NMR, ^{1}H coupled, δ (ppm) = −90.5, dd, vs $CFCl_3$) was still present after complete inactivation of the enzyme with the release of 2.2 mol of fluoride anion (^{19}F NMR, δ (ppm) = −120.4 vs $CFCl_3$) per mole of inactivated enzyme. In the experiment with TFMTA using a 7-fold excess of nucleoside per enzyme subunit, the remaining TFMTA was identified by its ^{19}F NMR signal (δ (ppm) = −39, vs $CFCl_3$) and the fluoride ion released, 3.1 mol per mole of inactivated enzyme, was determined from the ratio of the integration of ^{19}F NMR signals of F^- and TFMTA (Figure 3) as described in Methods.

Characterization of Reaction Products

Upon complete inactivation of the enzyme by labelled **1a** or **1b**, the reaction products were analyzed by radiochromatography. In addition to the

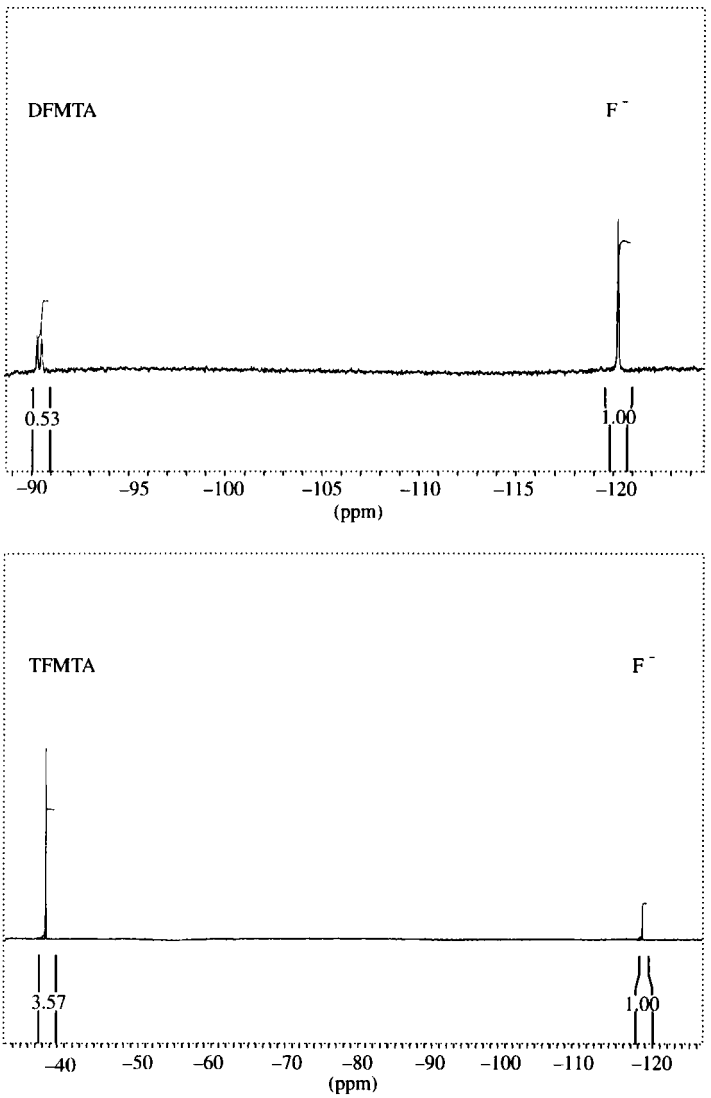


FIGURE 3 Determination of fluoride release from DFMTA **1a** and TFMTA **1b**. Spectra were obtained using a Brucker AC 250 (235 MHz for ^{19}F). Chemical shifts reported are relative to external fluorotrichloromethane. A total of 465 000 scans for DFMTA and 267 000 for TFMTA were collected (no pulse delay). A control was conducted without enzyme under the same conditions. No fluoride anion was detected.

unreacted [8-^{3}H]-DFMTA or [8-^{3}H]-TFMTA (characterized by their Rf values of 0.6, in ethyl acetate/MeOH: 80/20 as eluent), a new radioactive product appeared in the chromatogram. This compound which corresponded to adenine was identified by comparison of its migratory velocity

on TLC with an authentic sample of adenine, in various solvent systems and by HPLC analysis monitored under the conditions described in Methods using adenine as reference. In both cases no trace of labelled adenosine was detected.

DISCUSSION

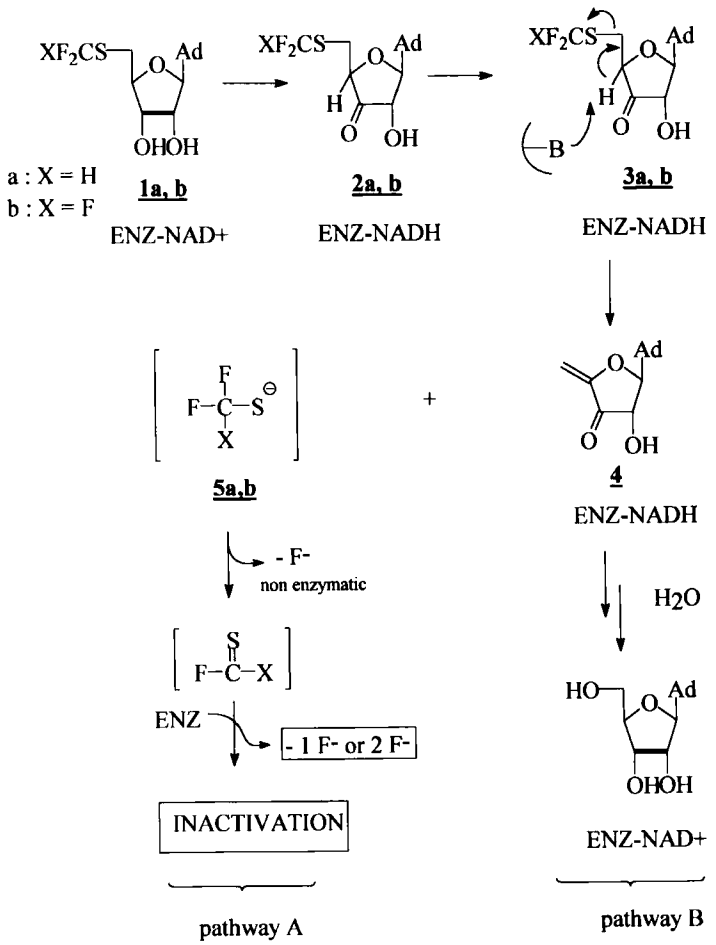
5′-Methylthioadenosine (MTA) and 5′-isobutylthioadenosine (SIBA) have been shown to irreversibly inactivate AdoHcy hydrolase.[12] Although the mechanism of action of MTA and SIBA on AdoHcy hydrolase has not been demonstrated, these nucleosides presumably act as type I mechanism-based inhibitors like some other 5′-substituted adenosine inhibitors of AdoHcy hydrolase.[15] This observation and the fact that difluoromethylthio and trifluoromethylthio moieties in the β position to a keto group can decompose to carbonothioic fluoride derivatives[16] led us to hypothesize that analogs of MTA fluorinated at the 5′-methylthio position, like **1a** and **1b** might serve as enzyme-activated acylating agents for AdoHcy hydrolase. According to the Palmer–Abeles[4] mechanism of AdoHcy hydrolase, if enzymatic oxidation of DFMTA or TFMTA could occur at the C-3′ position, the formation of their corresponding 3′-keto derivatives **2a, b** might favor the β-elimination of difluoromethyl or trifluoromethyl thiolate ions respectively (XCF_2S^-, X = H, F). These unstable species are known to decompose into highly reactive acylating agents such as thioformyl fluoride and carbonothioic difluoride (XFC = S, X = H, F). Generated in the enzyme cavity the latter could irreversibly acylate nucleophilic residues involved in the catalytic process[17] directly or through their hydrolysis products. This possibility has been examined with the two fluorinated analogs of MTA, **1a** and **1b**.

When beef liver AdoHcy hydrolase was incubated with DFMTA or TFMTA, activity was lost as shown in Figure 2. The first-order kinetics of inhibition, irreversibility and protection by substrate suggest that activity is lost by an enzyme-activated irreversible inhibition in both cases.

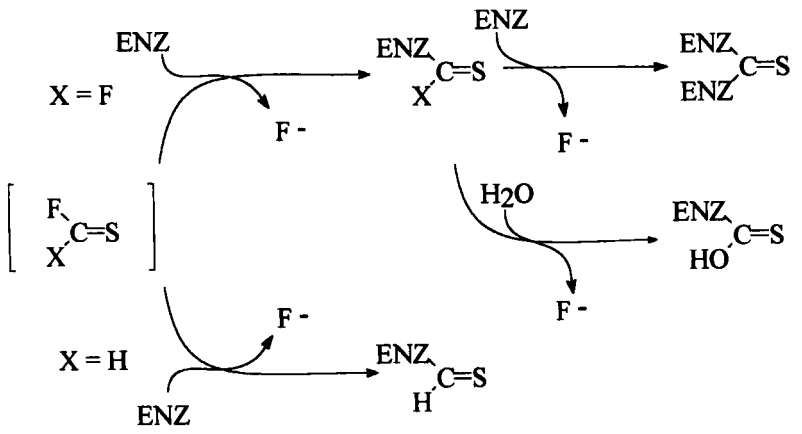
The presumed oxidation of **1a, b** to their corresponding 3′-keto derivatives **2a, b** by the enzyme during the inactivation process was indicated by an increase in the absorbance at 320 nm due to the putative formation of an ENZ–NADH complex by analogy with other irreversible inhibitors.[15] The increase in absorbance at 320 nm was maximum when the enzyme was completely inactivated by **1a** or **1b**, but the NADH formed was not quantitavely determinated in our experiments.

Involvement of a β-elimination step leading to 3′-keto-4′,5′-dehydroadenosine **4** and thioformyl fluoride or carbonothioic difluoride via their

corresponding difluoromethylthiolate and trifluoromethylthiolate ion intermediates is further supported by ^{19}F NMR spectroscopy experiments designed to detect the release of fluoride ion. As shown in Figure 3 when an excess of DFMTA or TFMTA was incubated with AdoHcy hydrolase, the release of 2.2 and 3.1 mol of fluoride anion per mole of inactivated enzyme was observed respectively. One equivalent of F^- was expected to be formed in each case from the decomposition of the unstable fluoromethylthiolate ions produced in the experiment. With **1a**, the formation of a second equivalent of fluoride ion could occur either from the reaction of the thioformyl



SCHEME 1 Mechanism of inactivation of AdoHcy hydrolase by DFMTA **1a** and TFMTA **1b**.



SCHEME 2 Possible inactivation process involving monofunctional or bifunctional acylating species generated from DFMTA **1a** or TFMTA **1b** by AdoHcy hydrolase.

fluoride formed with an active site nucleophile or by its hydrolysis to generate thioformic acid, which may diffuse. In the experiment in which **1b** was reacted with AdoHcy hydrolase the formation of difluorothiocarbonyl is expected. Interaction of this cross-linking agent[16] with enzyme could liberate two F^- equivalents. But direct hydrolysis of F_2CS to COS (or H_2S and HF) must also be considered (Scheme 2).

In the proposed mechanism of inactivation (Scheme 1), each catalytic event with **1a** and **1b** also produces 3′-keto-4′,5′-dehydroadenosine an intermediate which is converted by the normal enzymatic reaction into adenosine in the absence of homocysteine. If the highly reactive and hydrolysable acylating agents, CHF=S or CF_2=S produced in these two experiments are not immediately quenched by reaction with a nucleophile, adenosine might be formed. Since no adenosine was detected in solution upon incubation of excess of **1a** or **1b** with enzyme, each turnover must lead to inactivation. The formation of the adenine isolated in both experiments, might result from the decomposition of the accumulated unstable intermediate **4**.[4]

TFMTA appeared to be a better inactivator in terms of its k_{inact} value. This might reflect the more powerful electron withdrawing properties of the adenosine C-5′ trifluoromethyl group which might facilitate the cleavage of the C-4′ C–H bond and consequently the β-elimination step. It is more difficult to explain the large difference in the apparent binding constant K_i observed for these two nucleosides. Studies of steric hindrance of the CF_3 group have shown it to be as bulky as an isopropyl group.[18] Taking into account the K_i values of MTA (47 μM) and SIBA (42 μM) calculated for rat

liver AdoHcy hydrolase, by Della Ragione *et al.*[12] it seems that steric factors cannot explain this difference. It is more likely that the enzyme is sensitive to an electronic effect in that position since K_i is multiplied by a factor of 10 with just the addition of one fluorine atom.

In summary, the results of these investigations have revealed that DFMTA and TFMTA are potent inactivators of AdoHcy hydrolase and the main lines of their inactivation process have been determined. Only the precise nature of the acylating agent by which inactivation of the enzyme proceeds remains unknown.

DFMTA and TFMTA represent a new class of mechanism-based inhibitors for AdoHcy hydrolase. Their ability to acylate a nucleophilic residue involved in the catalytic process makes them valuable tools to determine the nature of this essential amino acid present at the active site of the enzyme. Work is in progress in this direction.

Acknowledgements

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