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5'-DEOXY-5'-DIFLUOROMETHYLTHIOADENOSINE, A POTENT ENZYME-ACTIVATED ACYLATING AGENT OF S-ADENOSYL-L-HOMOCYSTEINE HYDROLASE.

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Abstract : 5'-Deoxy-5'-difluoromethylthioadenosine (DFMTA) caused potent time-dependent inactivation of S-adenosyl-L-homocysteine hydrolase. The mechanism of this inactivation was investigated.
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The importance of S-adenosyl-L-homocysteine (AdoHcy) hydrolase (E.C.3.3.1.1) as a target for developing antiviral agents has been outlined [1a,b,c] and the design and synthesis of mechanism-based inhibitors have received considerable attention since the mechanism of catalysis of AdoHcy hydrolase was elucidated by Palmer and Abeles[2]. This mechanism involves oxidation of the 3'-hydroxyl group of AdoHcy by enzyme bound NAD^+, followed by β elimination of L-homocysteine to give an α,β insaturated ketone. Michael addition of water to the intermediate affords 3'-ketoadenosine which is then reduced by NADH to adenosine (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[3]. Type II mechanism-based inhibitors utilises the same oxidative action of the enzyme to generate an electrophilic site on the inhibitor, which can bind covalently with an active site nucleophile[4a,b].

This paper describes a novel approach to irreversible inhibition of AdoHcy hydrolase involving the use of an enzyme-activated acylating agent.

5'-Deoxy- 5'- methylthioadenosine (MTA) has been shown to inactivate irreversibly AdoHcy hydrolase[5]. Although the mechanism of action of MTA on AdoHcy hydrolase has not been demonstrated, this nucleoside presumably acts as type I mechanism-based inhibitor like some other 5'-subtituted adenosine inhibitors of AdoHcy hydrolase[6]. This observation and the fact that difluoromethylthio and trifluoromethylthio moieties in the β position to the keto group can decompose to carbonothioic fluoride derivatives (XFC=S, X = H, F)[7a,b] led us to hypothesize that analogs of MTA fluorinated at the 5'-methylthio position might serve as a novel type of irreversible inhibitors of AdoHcy hydrolase. This possibility was first examined with 5'-deoxy-5'-(difluoromethylthio) adenosine (DFMTA), because this nucleoside revealed interesting growth inhibitory properties, although its sites of action have not yet been fully explored[8a,b]. Based on the Palmer - Abeles mechanism of AdoHcy hydrolase, enzymatic oxidation of DFMTA **1** to the 3'-keto derivative **2** if occured could be accompanied by a β-elimination of difluoromethylthiolate ion (CHF_2S^-), leading to thioformyl fluoride

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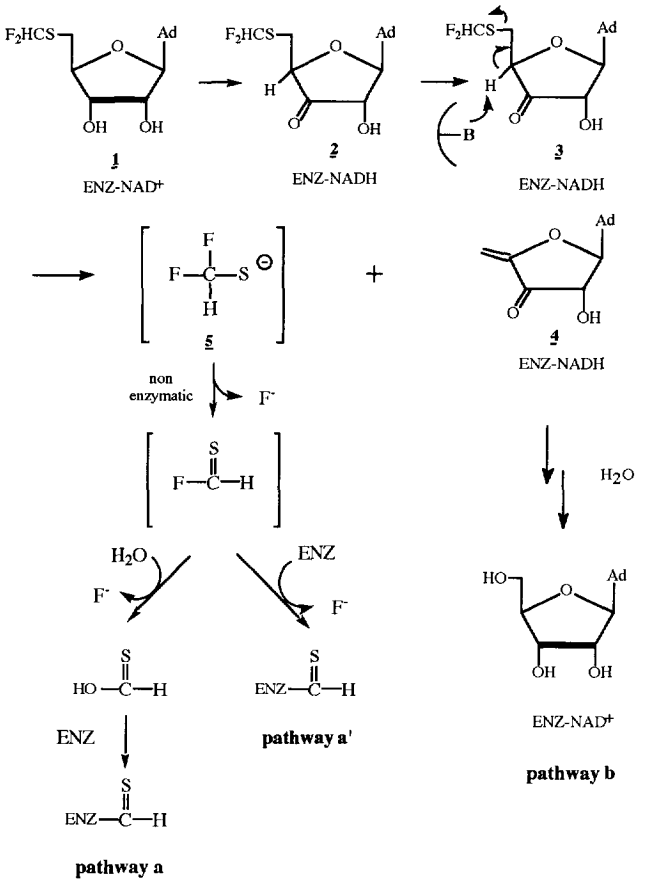
(HFC=S) within the enzyme cavity (scheme 1). The latter could irreversibly alkylate nucleophilic residues[9a,b] involved in the catalytic process directly, due to its highly electrophilic character (pathway a') or through its hydrolysis product, thioformic acid (pathway a).



Scheme 1 : mechanisms of conversion of DFMTA by AdoHcy hydrolase

DFMTA was prepared from adenosine according to the general procedure described by Honek and coworkers[8b], via a 5'-deoxy-5'-thioacetate adenosine derivative. Pure DFMTA obtained this way was assayed for its activity on AdoHcy hydrolase, purified to homogeneity from rat livers as described by Kajander and Raina[10]. The enzyme used in our experiments had a specific activity of 4 units/mg. AdoHcy hydrolase activity was assayed in the direction of AdoHcy synthesis using (8-^{14}C)-Adenosine as described previously[11]. DFMTA is a remarkably stable compound and no decomposition of this nucleoside occured in the buffer assay in the absence of the enzyme.

Incubation experiments with AdoHcy hydrolase and DFMTA showed a time-dependent loss of enzyme activity, the deactivation rate depending on the inhibitor concentration (Fig 1a). Pseudo first-order kinetics were observed and from a reciprocal plot of apparent rate constants (K_{app}) against reciprocals of inhibitor

concentrations (Fig 1b), according to the method of Kitz and Wilson[12], the following inhibition parameters were estimated : K_{inact} = 0.07 min $^{-1}$ and K_i = 5.2 µM (K_m Ado = 1µM).

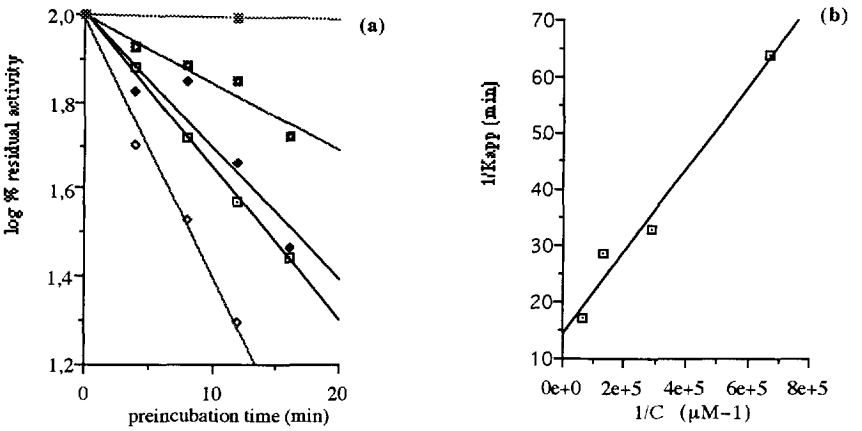


Fig 1 (a) : Time and concentration dependent inactivation of AdoHcy hydrolase by 1 .6 nM Rat liver AdoHcy hydrolase was preincubated for the indicated times at 37°C with different concentrations of 1. Assays performed with : 8-^{14}C Ado [10 µM], Hcy [5 mM], 20mM potassium phosphate buffer pH 7.5, 1mM EDTA, 1 mg/mL BSA and 1 (1.8, 4.0, 8.0 , 18.0 µM).

(b) : Plot of $1/K_{app}$ vs 1/[1] from which the K_i and k_{inact} values were calculated.

A time dependent increase in absorbance at 340 nm was observed when DFMTA was mixed with enzyme (UV spectrum not shown), which is consistant with the formation of enzyme-bound NADH and oxidation of the 3'-hydroxyl of DFMTA by analogy with oxidation of other irreversible inhibitors.

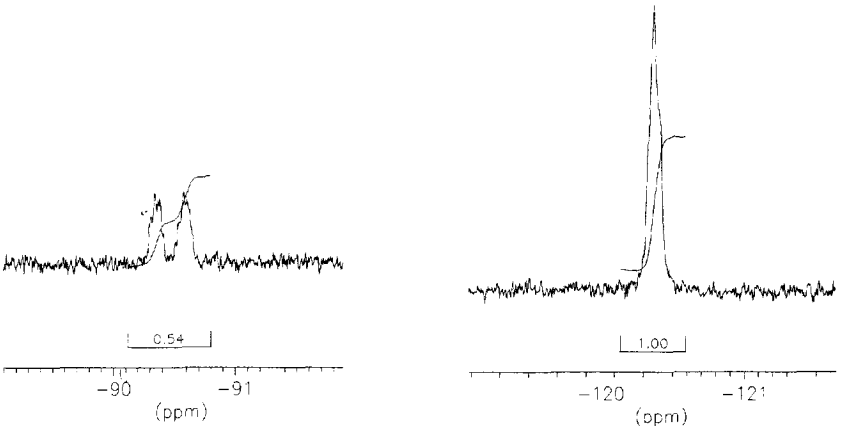


Fig 2 : AdoHcy hydrolase (1.4 10^{-7} mole) was incubated in phosphate potassium buffer 20 mM pH 7.5 (3.5 mL) with 1 (4.0 10^{-7} mole) at 37 °C. After total inactivation, ethanol (9 mL) was added, the mixture centrifuged, concentrated , dissolved in D_2O (0.5 mL) and analysed by ^{19}F NMR spectroscopy. Spectra were obtained using a Brucker AC 250 (235 MHz for ^{19}F). Chemical shifts reported are relative to external fluorotrichloromethane. A total of 465 436 scans (no pulse delay) were collected. The number of mole 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/2 and fluoride signal)] x moles of inhibitor used in the experiment. A control was made in the same conditions of buffer without enzyme, no fluoride anion was detected.

Finally, the proposed scheme of inactivation of AdoHcy hydrolase involving a β-elimination step leading to 3'-keto-4',5'-dehydroadenosine **4** and thioformyl fluoride (via a difluoromethylthiolate ion) is further supported by the release of fluoride anion from DFMTA. As shown in Fig 2, when a 3 fold excess of DFMTA per enzyme subunit was incubated with purified AdoHcy hydrolase, unreacted DFMTA was shown to be still present after complete inactivation of enzyme (^{19}F NMR, δ_{ppm} = - 90.5, dd, vs $CFCl_3$) with the release of 2.2 mol of fluoride anion per mol of inactivated enzyme (^{19}F NMR, δ_{ppm} = - 120.4 vs $CFCl_3$). One equivalent of inorganic fluoride was expected to be formed from the decomposition of the unstable difluoromethylthiolate ion. The formation of a second equivalent of fluoride ion could occur either by hydrolysis of thioformyl fluoride to generate thioformic acid in the active site or by reaction of the thioformyl fluoride with an active site nucleophile.

In the normal reaction, the enzyme catalyses the addition of water to the enone **4**, in our experiment no adenosine was detected in the incubation mixture. This result indicates that the inhibition process (pathway a or a') is largely predominant in this experiment, but the nature of the acylating agent by which inactivation of the enzyme proceeds remains unknown.

Although this concept of freeing little electrophilic entity within an enzyme has already been applied, to our knowledge, this has never been illustrated with AdoHcy hydrolase. Investigations with mono and trifluoromethylthioadenosine are currently in progress.

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