S-Adenosylhomocysteinase: Mechanism of Inactivation by 2′-Deoxyadenosine and Interaction with Other Nucleosides†

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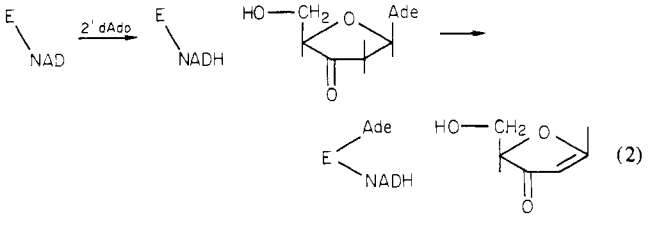
ABSTRACT: *S*-Adenosylhomocysteinase (SAHase), a tetrameric enzyme, is inactivated by 2′-deoxyadenosine (2′dAdo) in a time-dependent process [Hirshfield, M. S. (1979) *J. Biol. Chem. 254*, 22–25]. It has been proposed that inactivation involves oxidation of 2′dAdo at C-3′ by enzyme-bound nicotinamide adenine dinucleotide (NAD), subsequent proton abstraction at C-2′, and elimination of adenine. This results in irreversible formation of enzyme-bound NADH and of adenine (Ade) and inactivation [Abeles, R. H., Tashjian, A. H., Jr., & Fish, S. (1980) *Biochem. Biophys. Res. Commun. 95*, 612–617]. It has now been established that upon inactivation of SAHase with deoxy[2′(*R*)-^{3}H]adenosine, ^3H_2O is formed. This is consistent with the proposed mechanism and establishes that a trans elimination occurs. The stoichiometry of ^3H_2O release shows that maximally two of the four subunits participate in the reaction that results in ^3H_2O release. Reaction of SAHase with 2′dAdo results in reduction of two of the enzyme-bound NAD molecules. However, all four NAD molecules can be reduced by $NaBH_4$, but only two are reduced to C-4 NADH. When the enzyme is inactivated with adenine-labeled 2′dAdo, radioactivity corresponding to 0.5–1.0 μmol of 2′dAdo binds tightly per micromole of subunit. This radioactive material is not removed from the enzyme by extensive dialysis but can be displaced by unlabeled 2′dAdo or Ade. After denaturation of the complex, radioactive material is released. Of this material 80–90% is adenine and less than 1% 2′dAdo. 2′dAdo also binds tightly to the enzyme reduced with $NaBH_4$. Upon denaturation mostly adenine (80–90%) is released. Reaction of [2′-^{3}H]2′dAdo with enzyme reduced with $NaBH_4$ does not result in ^3H_2O formation. We conclude that the enzyme catalyzes the release of adenine from 2′dAdo by two mechanisms: One involves formation of 3′-keto-2′dAdo and subsequent elimination of adenine. The other does not involve oxidation of 2′dAdo and probably is a hydrolytic process. It is proposed that the ability of the enzyme to carry out the hydrolytic process is a direct consequence of the manner in which 2′dAdo as well as the normal substrate binds to the enzyme, i.e., hydrogen-bond interaction of the protein with the adenine moiety and distortion of the ribose ring. When adenine-labeled adenosine is added to the enzyme, radioactivity corresponding to 0.5 μmol/μmol of subunit is associated with the protein after gel filtration. Of the radioactive material bound to the protein, 20% is adenine, 15% is adenosine, and the remaining radioactivity is present in unidentified compounds. The adenine bound to the enzyme does not participate in the catalytic process, and we conclude that it is bound to two of the subunits that do not participate in catalysis. Possibly, these two subunits have a regulatory function. SAHase probably consists of two nonequivalent pairs of subunits. Only one pair participates in catalysis, but all four subunits probably bind Ado and 2′dAdo. We have confirmed the fact that the carbocyclic analogue of adenosine inactivates SAHase [Guranowski, A., Montgomery, J. A., Cantoni, G. L., & Chiang, P. K. (1981) *Biochemistry 20*, 110–115]. Inactivation is accompanied by reduction of enzyme-bound NAD. After denaturation of the inactivated enzyme, the major compound isolated is adenine.

S-Adenosylhomocysteinase (SAHase)[1] catalyzes the reaction shown in eq 1. The enzyme is inactivated in a time-

$$\text{SAH} \rightleftharpoons \text{adenosine} + \text{homocysteine} \qquad (1)$$

dependent process by 2′dAdo (Hirshfield, 1979). A mechanism has been proposed for this inactivation (eq 2) (Abeles



(2)

et al., 1980) that closely parallels the normal catalytic process. In both reactions the substrate, 2′dAdo in the inactivation reaction and Ado in the catalytic process, is initially oxidized at C-3′ with the concomitant reduction of enzyme-bound NAD. Subsequently the processes diverge. With Ado, the normal substrate, elimination of H_2O occurs between C-4′ and C-5′ whereas with 2′dAdo, Ade is eliminated between C-1′ and C-2′ (eq 2). Both elimination reactions are facilitated by the carbonyl group at C-3′. In the reaction leading to inactivation, adenine elimination occurs due to the intrinsic instability of 3′-keto-2′-deoxyadenosine (Pfitzner & Moffat, 1963). Inactivation of the enzyme is the consequence of the formation of enzyme–NADH, since E–NAD is required for the catalytic process. The possibility that the dehydro keto sugar could covalently modify the enzyme was also considered (Abeles et al., 1980).

In a previous paper (Abeles et al., 1980), we have presented the following facts in support of the proposed inactivation mechanism: (1) Enzyme-bound NADH is formed concomi-

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[1] Abbreviations: 2′dAdo, 2′-deoxyadenosine; SAHase, *S*-adenosylhomocysteinase; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine; SAH, *S*-adenosylhomocysteine; NAD, nicotinamide adenine dinucleotide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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tant with inactivation. (2) Upon denaturation of SAHase, inactivated with 2′dAdo, Ade is released. No 2′dAdo can be detected. No data concerning the fate of the 2′-deoxyribose moiety of 2′dAdo are available. (3) Ade is extremely tightly bound to the inactivated enzyme; i.e., it is not removed by gel filtration or prolonged dialysis.

In this paper, we report additional evidence for the proposed inactivation mechanism. We also have investigated the stoichiometry of the interaction between 2′dAdo and SAHase, including the question of subunit equivalence. Additionally, we have investigated the interaction of SAHase with other nucleosides.

Materials and Methods

Reagents and Radioactive Compounds. All radioactive materials were purchased from New England Nuclear Corp. except [8-^{3}H]2′dAdo, which was purchased from ICN, and were purified prior to use on TLC. Adenosine deaminase and alcohol dehydrogenase were purchased from Sigma Chemical Co. Carbocyclic adenosine was a gift from Dr. John Gerlt, and EHNA was a gift from Dr. Gertrude Elion. All other reagents were obtained from commercial sources and used without further purification.

Reaction of SAHase with 2′dAdo. The reaction mixture contained 0.5 mg of SAHase, 1 mM K-EDTA, 100 μg of EHNA, 6.6 mM 2′dAdo, 1 mM DTT, and 25 mM potassium phosphate buffer, pH 7.0, in a total volume of 150 μL. EHNA was added to prevent deamination of 2′dAdo by traces of contaminating adenosine deaminase. The reaction was carried out in most cases with 2′dAdo labeled in the adenine moiety. In a few experiments, 2′dAdo uniformly labeled with ^{14}C was used. The reaction was allowed to proceed for 90 min at 22 °C. At this point, at least 90% of the enzyme activity was lost. The solution was placed on a Bio-Gel P-6 column (100–200 mesh), 0.8 × 23 cm, equilibrated with buffer A. The column was eluted with the same buffer, and 0.5-mL fractions were collected. The enzyme eluted with 5–6 mL of buffer and 2′dAdo with 16–20 mL. Radioactivity eluted with protein. The complex obtained at this point will be referred to as SAHase–2′dAdo. To the fraction containing the protein (1.0 mL) was added 100 μL of 2 N $HClO_4$ at 4 °C. The precipitate was discarded. The supernatant fluid was neutralized with 5 N KOH. The resulting precipitate was removed by centrifugation, and the supernatant fluid was concentrated to 300 μL with a stream of N_2. HPLC analysis was performed on this concentrate. At this point, 85–90% of the radioactive material present in the protein fraction was recovered. Buffer A consists of 25 mM potassium phosphate buffer, pH 7.0, 1 mM DTT, and 1 mM K-EDTA. TLC analysis was carried out on Eastman cellulose sheets (13245) with *n*-BuOH–MeOH–H_2O–NH_4OH, 60:20:20:1. HPLC was performed on a Waters μBondapak C_{18} column, 7.8 × 30 cm, with 13% MeOH in H_2O as eluent.

Determination of Enzyme-Bound NAD and NADH. The reaction mixture contained 0.1 mL of enzyme (0.63 mg), 0.05 mL of 50 mM 2′dAdo, or 0.05 mL of H_2O. The reaction proceeded for 90 min. At this point less than 10% of the enzyme activity remained. The reaction was stopped by the addition of 2 volumes of 95% ethanol. The precipitate was removed and washed with 1 volume of ethanol. The combined supernatant fluids were brought to dryness with a stream of N_2. The residue was taken up in 1.0 mL of H_2O, and aliquots were used for analysis. NAD was determined with liver alcohol dehydrogenase (Klingenberg, 1974). The following reaction mixture was used for the NADH assay: 0.1 M Tris-HCl, pH 7.3, 0.01 M glycolaldehyde, 100 μg of liver alcohol dehydrogenase, and aliquots to be assayed or NADH standard, in a total volume of 1.0 mL at 25 °C. The decrease in absorbance at 340 nm was determined. The HPLC assay was done with a Whatman SAX-10 column, 4.6 × 250 mm. The initial elution was isocratic with 3 mM potassium phosphate, pH 5.8. After 20 min, a gradient was started with 150 mM potassium phosphate, pH 6.5, containing 250 mM KCl. The gradient reached 100% of the 150 mM buffer after 60 min. The flow rate was 2 mL/min. NAD eluted after 7.5 min and NADH after 39 min.

Synthesis of [2′(R)-^{3}H]2′dAdo. The following were put in a small test tube: 0.1 mL of 1 M Tris-HCl, pH 7.3, 0.01 mL of 0.3 M NaEDTA, and 0.02 mL of 0.05 M dGTP. The reaction mixture was brought to dryness under vacuum, and the following were added: 1.0 mL of ^3H_2O (1 Ci/mL), 0.05 mL of 0.5 M Tris–ATP, 3 mg of DTT, 0.1 mL of ribonucleotide reductase[2] (the amount of this enzyme preparation required was empirically determined), 0.015 mL of 1 N NaOH, and 0.01 mL of 1 mM B_{12} coenzyme. The coenzyme was added in the dark, and the subsequent reaction was carried out in the dark. The reaction vessel was flushed with N_2, and the reaction was carried out at 37 °C. Periodically aliquots were removed to monitor the progress of the reaction. Aliquots (0.005 mL) of the reaction mixture were added to the following: 0.01 mL of 1 M Tris-HCl, pH 7.3, 0.09 mL of H_2O, and 2 μL of *Escherichia coli* alkaline phosphatase (0.6 IU, Sigma type III). The reaction proceeded at 37 °C for 15 min. It was then placed in a boiling water bath for 5 min. The denatured protein was removed, and an aliquot of the supernatant fluid was assayed by HPLC to determine the amount of 2′dAdo formed. When at least 80% of the ATP was converted to dATP, 0.025 mL of alkaline phosphatase was added. The reaction was allowed to proceed at 37 °C for 15 min and then placed in a boiling water bath for 5 min. The reaction mixture was bulb-to-bulb distilled 3 times. The residue was taken up in 1.0 mL of H_2O and insoluble material removed by centrifugation. Successive 0.05-mL aliquots of the supernatant were purified on a Waters μBondapak C_{18} column. A solution of 13% methanol in H_2O was used as eluent. The fractions containing 2′dAdo were combined, brought to dryness, and redissolved in 1.0 mL of H_2O. The purity of the resulting [2′-^{3}H]2′dAdo was assayed by chromatography on HPLC. More than 97% of the radioactive material comigrated with 2′dAdo. The specific activity of 2′dAdo was 1.9 × 10^6 cpm/μmol.

S-Adenosylhomocysteinase. The enzyme was isolated from calf liver (Richards et al., 1978). The crystallization step was omitted. The enzyme was >95% pure as judged by NaDodSO$_4$ gel electrophoresis. The enzyme concentration was determined from the absorbance at 280 nm; $E^{1\%}_{1cm}$ = 14.2. For calculation of subunit concentration, a subunit molecular weight of 48 × 10^3 was used (Palmer & Abeles, 1979). The enzyme was assayed by spectrophotometry or by the tritium-exchange method (Palmer & Abeles, 1979).

Reduction of S-Adenosylhomocysteinase with $NaBH_4$. To 0.2 mL of a solution of SAHase (1–2 mg) in 0.01 M potassium phosphate buffer, pH 7.4, 1 mM K-EDTA, and 0.2 mM DTT was added 50 μL of a $NaBH_4$ solution (2 mg of $NaBH_4$/mL of 0.01 M NaOH). The reaction was allowed to proceed for 15–30 min at room temperature. At this point the enzyme was completely inactivated (Palmer & Abeles, 1979). The reaction mixture was dialyzed for 12 h against 100 mL of the

[2] Ribonucleotide reductase from *Lactobacillus leichmanii* was a generous gift from Professor R. Blakely.

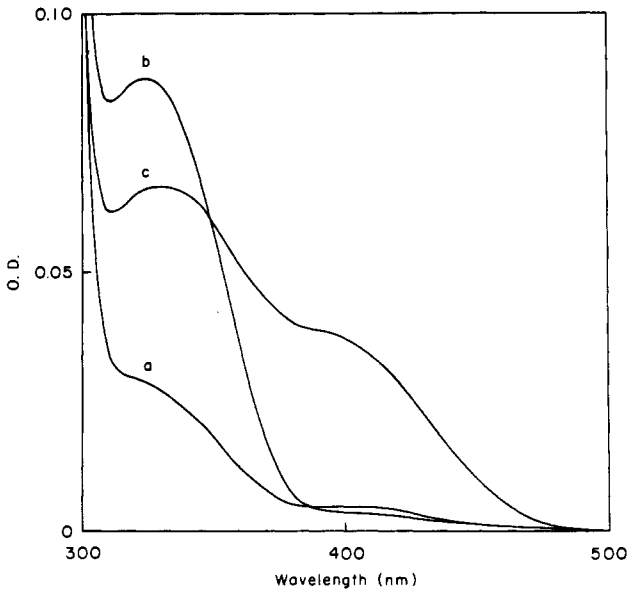

FIGURE 1: Spectra of *S*-adenosylhomocysteinase. The reaction mixture contained 0.97 mg of SAHase, 10 mM potassium phosphate, pH 7.4, 1 mM K-EDTA, and 100 μg of EHNA in a total volume of 1.0 mL. (a) No addition; (b) 2.5 mM 2′dAdo; (c) enzyme reduced with $NaBH_4$ in place of native enzyme. Spectra were taken with a Perkin-Elmer 559 spectrophotometer at 25 °C against a reference cell containing all components except enzyme.

Table I: NAD and NADH Content of SAHase

	μmol/μmol of enzyme subunit			
	NAD[a]	NADH[a]	NAD[b]	NADH[b]
enzyme	1.1	0	0.9	0.1
enzyme + $NaBH_4$	0	0.4	0.1	0.5
enzyme + 2′dAdo	0.4	0.6		

[a] NAD and NADH determined with alcohol dehydrogenase. [b] NAD and NADH determined by HPLC. Experimental conditions are described under Materials and Methods.

buffer in which the enzyme was originally dissolved.

Results

Reaction of Enzyme-Bound NAD with 2′dAdo and $NaBH_4$. SAHase was inactivated with 2′-deoxyadenosine (Hirshfield, 1979) and with $NaBH_4$ (Palmer & Abeles, 1979). The spectra obtained after inactivation are shown in Figure 1. The addition of 2′dAdo leads to the formation of enzyme-bound NADH (Abeles et al., 1980). Only two of the four NAD molecules are reduced. (This conclusion is based on the assumption that the extinction coefficient of enzyme-bound NADH is the same as that for NADH in solution.) The spectrum obtained after the addition of $NaBH_4$ differs from that obtained in the presence of 2′dAdo, and therefore, reaction with $NaBH_4$ must lead to the formation of a compound(s) other than C-4 NADH. The spectrum of the enzyme reduced with $NaBH_4$ closely resembles the spectrum obtained when NAD in solution is reduced with $NaBH_4$. $NaBH_4$ reduction in solution leads to a mixture of 1,4-, 1,2-, and 1,6-NADH (Chaykin & Meissner, 1964). The amount of NAD and NADH associated with the native and inactivated enzyme was determined after denaturation of the enzyme. The results are summarized in Table I. Consistent with previous results (Palmer & Abeles, 1979), the native enzyme contains close to 1 μmol of NAD/μmol of subunit. Inactivation with 2′dAdo leads to the reduction of approximately 50% of the enzyme-bound NAD. The amount of NADH found in a number of experiments ranges from 0.4 to 0.6 μmol/μmol of subunit. With $NaBH_4$ all of the NAD disappears and presumably is reduced. However, only approximately 50% of the NAD can be accounted for as NADH by HPLC or assay with alcohol dehydrogenase. We suggest that 50% of the NAD is reduced in the 4 position and 50% in the 2 position. The latter is not enzymatically detectable and decomposes in the phosphate buffer used for chromatrography (Chaykin & Meissner, 1964). The results show that the four NAD molecules of the tetrameric enzyme are not equivalent. The nonequivalence of the two pairs of subunits is indicated by the results obtained with 2′dAdo. Addition of 2′dAdo leads to reduction of two of the four NAD molecules. The spectral change observed in the presence of normal substrate, Ado, is very similar to that obtained in the presence of 2′dAdo. Therefore, in the catalytic process at steady state, two of the four NAD molecules are reduced, and it is very probable that only two subunits participate in the catalytic process.

Products Derived from 2′dAdo. SAHase was inactivated with [8-^{3}H]2′dAdo, and SAHase–2′dAdo was isolated (see Materials and Methods). The radioactive material derived from 2′dAdo associated with SAHase–2′dAdo is tightly bound. Dialysis against two changes of 200 volumes of buffer A for 48 h resulted in the loss of 14% of the protein-bound radioactivity. The amount of radioactivity bound to the enzyme varied with different enzyme preparations. With all enzyme preparations, under standard inactivation conditions, minimally 88% of the catalytic activity is lost, and radioactivity corresponding to at least 0.5 μmol of 2′dAdo/μmol of enzyme subunit is associated with the protein. Maximally stoichiometric amounts of radioactive material are bound to the enzyme. With enzyme preparations in which less than stoichiometric binding occurs, under standard conditions, prolonged incubation of the enzyme with 2′dAdo increases the amount of radioactive material bound. Therefore, the observation that under a fixed set of experimental conditions, different amounts of 2′dAdo react per mole of enzyme reflects differences in rate of reaction between 2′dAdo and enzyme rather than in intrinsic differences in stoichiometry.

In order to identify the radioactive compound(s) associated with SAHase–2′dAdo, it was necessary to denature the complex with $HClO_4$ or ethanol. Identical results were obtained with both procedures. Of the released radioactive material, 80–90% cochromatographed with adenine on HPLC, as well as TLC. In addition to adenine, two or three other radioactive compounds were detected by HPLC. One radioactive peak, which constituted 0–10% of the radioactive material applied to the column, eluted with the same retention time as 2′-deoxyinosine. However, the material was not further characterized. No attempt was made to identify the other two peaks. 2′dAdo was not found; i.e., it constituted less than 1.0% of the total radioactivity.

The data presented above establish that 4 mol of 2′dAdo can react with 1 mol of tetrameric enzyme and that 80–90% of the 2′dAdo is converted to Ade and an as yet unidentified compound derived from the deoxyribose moiety. However, only 2 mol of NADH are formed per mole of tetrameric enzyme. Therefore, not all of the 2′dAdo that reacts with SAHase is oxidized, and a mechanism exists for Ade release from 2′dAdo that does not involve oxidation of 2′dAdo.

SAHase–2′dAdo was prepared with [U-^{14}C]2′dAdo in order to determine whether the 2-deoxyribose moiety is part of the complex. The specific activity (cpm per micromole of subunit) of the complex derived from [U-^{14}C]2′dAdo was 50% that of the complex derived from [8-^{3}H]2′dAdo. It is, therefore, very

likely that the deoxyribose moiety is not retained in the complex.

We next examined the effect of $NaBH_4$ reduction of the enzyme on the binding of adenine-labeled 2′dAdo. The enzyme used in these experiments was catalytically inactive and contained only reduced forms of NAD. The reaction of 2′dAdo with $NaBH_4$-inactivated enzyme and the subsequent isolation of the complex were carried out as described for the native enzyme. The amount of radioactivity bound to the enzyme varied with different enzyme preparations and ranged from 0.5 to 1.0 μmol of 2′dAdo/μmol of enzyme subunit. Upon denaturation of the complex, from 80% to 90% of the enzyme-bound radioactivity was present as adenine. These results confirm the conclusion reached above that adenine formation does not necessarily require the reduction of NAD; i.e., the adenine can be formed without prior oxidation of 2′dAdo.

The results obtained indicate that adenine derived from 2′dAdo does not dissociate from the enzyme. The interaction of adenine with enzyme was determined. Enzyme (0.36 mg) was incubated with [8-^{14}C]adenine (5.2 mM) under standard conditions for 1 h and then passed through a Bio-Gel P-6 column. From the radioactivity associated with the protein peak, it was determined that 0.05 μmol of adenine was present per micromole of subunit. A similar experiment was carried out with enzyme reduced with $NaBH_4$. With that enzyme, 0.9 μmol of adenine was bound per micromole of subunit. Thus, adenine binds strongly to enzyme containing NADH, consistent with the observation that adenine, derived from 2′dAdo, is not released from enzyme inactivated with 2′dAdo.

An experiment was then carried out to determine whether the radioactive material derived from 2′dAdo could be displaced from the enzyme by 2′dAdo. SAHase–2′dAdo was prepared with the following modifications: 2.65 mg of SAHase and 2.0 μmol of [8-^{3}H]2′dAdo (sp act. 14.6×10^6 cpm/μmol) were added to the reaction mixture in a final volume of 750 μL. The reaction was allowed to proceed for 3 h at 22 °C. SAHase–2′dAdo was then isolated and contained radioactive material corresponding to 0.77 μmol of 2′dAdo/μmol of enzyme. The solution of SAHase–2′dAdo was then divided into three aliquots of 650 μL each. To each aliquot was added 100 μg of EHNA in 10 μL of H_2O. To aliquot 1 was added 20 μL of H_2O. To aliquots 2 and 3 was added 20 μL of 50 mM 2′dAdo (nonisotopic). Aliquot 3 was immediately passed through a Bio-Gel P-6 column at 4 °C. The other two aliquots were kept at 22 °C for 2 h and then passed through the Bio-Gel column. The amount of radioactive material derived from 2′dAdo associated with the protein was determined. Aliquot 1 contained 0.72 μmol/μmol of enzyme subunit; i.e., no loss of enzyme-bound radioactivity occurred as a result of a 2-h incubation or subsequent manipulation. Aliquot 3 also contained 0.72 μmol/μmol of enzyme subunit. Therefore, displacement of radioactive material from the complex by 2′dAdo is not instantaneous. Aliquot 2 contained 0.57 μmol of radioactive material derived from 2′dAdo. These results show that addition of 2′dAdo to SAHase–2′dAdo displaces radioactive material from the complex in a time-dependent process. We also examined the fractions containing small molecules after filtration of aliquot 2 through the Bio-Gel column. This fraction contained radioactive material equivalent to 75% of the radioactive material displaced by unlabeled 2′dAdo from SAHase–2′dAdo. The radioactive material in the small-molecule fraction was examined by HPLC. Three major compounds were detected. A peak corresponding to 50% of the applied radioactive material cochromatographed with adenine, to 35% with deoxyinosine, and to 3% with 2′dAdo.

Another experiment was carried out in which the time of exposure of SAHase–2′dAdo to 2′dAdo (unlabeled) was varied. The experiment was carried out as was the experiment described above, except that initially adenine-labeled 2′dAdo was allowed to react with the enzyme for 1 h. The resulting SAHase–2′dAdo complex contained radioactive material corresponding to 0.5 μmol of 2′dAdo/μmol of subunit. When this complex was exposed to 2′dAdo (1.5 mM) for 2 h, 16% of the radioactive material was released; after 15 h 46% was released. The composition of the material released was as follows: 2′dAdo, 9%; adenine, 56%; material that cochromatographed with deoxyinosine, 33%. These experiments establish that compounds that do not dissociate from SAHase–2′dAdo can be displaced in a time-dependent process by 2′dAdo.

An analogous displacement experiment to those described above was carried out, except that SAHase–2′dAdo was incubated with adenine (1.5 mM) or hypoxanthine (1.5 mM) in place of 2′dAdo for 3 h. Adenine displaced radioactivity from SAHase–2′dAdo to the same extent as did 2′dAdo. Less than 6% of the radioactivity associated with SAHase–2′dAdo was displaced by hypoxanthine.

Interaction of SAHase with Other Nucleosides. [2-^{3}H]-Adenosine (6.6 mM) was incubated with SAHase for 90 min under standard conditions. The reaction mixture was then passed through a Bio-Gel P-6 column. Radioactive material was associated with the protein fractions. The amount of radioactivity with different enzyme preparations ranged from 0.5 to 0.7 μmol of adenosine/μmol of enzyme subunit. In no case did we observe stoichiometric binding. This was true of the enzyme preparation that bound 2′dAdo stoichiometrically. In all cases, the enzyme was fully catalytically active. Upon denaturation approximately 20% of the enzyme-bound radioactivity cochromatographed on HPLC with adenine and 15% with adenosine. The remaining radioactive material was found in unidentified peaks. No experiments were done to determine whether prolonged incubation increases the percentage of adenine found. It has been reported by others (Ueland & Helland, 1980; Saebo & Ueland, 1979; Ueland & Saebo, 1979) that SAHase from mouse liver binds adenosine tightly and catalyzes the formation of adenine and ribose from adenosine. The enzyme from lupin seed also binds 1 mol of adenosine/2 mol of subunit (Jakubowski & Guranowski, 1978).

To the enzyme–adenosine complex that had been passed through the Bio-Gel P-6 column was added unlabeled adenosine (10 mM). After 60 min, the enzyme was again subjected to gel filtration, and 82% of the radioactive material remained with the protein. In another experiment homocysteine (20 mM) and adenosine (10 mM) were added to the enzyme–adenosine complex that had been passed through the first Bio-Gel column. The reaction was allowed to proceed for 60 min. The enzyme was then again filtered through a Bio-Gel P-6 column, and 61% of the original radioactivity was found with the protein. These results show that adenosine that is tightly bound to enzyme does not participate in the catalytic process.

Carbocyclic adenosine binds tightly to SAHase ($K_i = 10^{-9}$) (Guarnowski et al., 1981) in a time-dependent process and inhibits the catalytic reaction. We have confirmed time-dependent inhibition. Carbocyclic adenosine (0.15 mM) was added to SAHase (0.65 mg) in 10 mM potassium phosphate buffer, pH 7.4, at 25 °C in a total volume of 1.0 mL. Addition of carbocyclic adenosine resulted in a spectral change that

Table II: ^3H Release from 2′-Deoxy[2′-^3H]adenosine[a]

		μmol of radioactive compound/ μmol of enzyme subunit	μmol of ^3H_2O/ μmol of enzyme subunit
1	enzyme + [2′-^3H]2′dAdo	0.05	0.4
2	$NaBH_4$-reduced enzyme + [2′-^3H]2′dAdo	0.05	0
3	enzyme + [8-^{14}C]2′dAdo	0.7	
4	$NaBH_4$-reduced enzyme + [8-^{14}C]2′dAdo	0.4	

[a] 0.48 mg of enzyme, 0.1 mg of EHNA, 6.6 mM [8-^{14}C]2′dAdo, in a total volume of 0.15 mL of buffer A, were allowed to react at 22 °C for 60 min. SAHase–2′dAdo was isolated as described under Materials and Methods; the amount of radioactive material bound to the enzyme was determined. A parallel experiment was carried out with [2′-^3H]2′dAdo. These reaction mixtures were bulb-to-bulb distilled under vacuum at the end of the reaction. The distillate was again bulb-to-bulb distilled, and the specific radioactivity of the distillate was determined. An aliquot of the distillate was also examined by HPLC (Waters μBondapak C_{18} 7.8 × 300 mm column, H_2O as eluent). A single radioactive peak was detected that had the same retention time as a ^3H_2O standard.

indicated formation of 0.5 μmol of NADH/μmol of subunit. The SAHase–carbocyclic adenosine complex was isolated by gel filtration as in other experiments. The enzyme was then denatured by addition of $HClO_4$ and the supernatant fluid examined by HPLC. No carbocyclic adenosine could be detected. The only UV-absorbing material found was adenine. From the amount of adenine detected, it can be determined that at least 90% of the enzyme-bound carbocyclic adenosine is converted to adenine and an unidentified product derived from the deoxyribose moiety.

Release of the 2′ Hydrogen of 2′dAdo as H_2O. The mechanism of inactivation of SAHase by 2′dAdo (eq 1) predicts that if the inactivation is carried out with [2′-^3H]-2′dAdo, $^3H_2$ will be formed. An experiment was done to verify this point. The results are summarized in Table II. When SAHase is incubated with [2′(*R*)-^3H]2′dAdo, radioactive material equivalent to 0.05 μmol of 2′dAdo/μmol of enzyme subunit is bound to the protein. ^3H_2O equivalent to 0.4 μmol of 2′dAdo/μmol of enzyme subunit is formed (experiment 1, Table II).

When an analogous experiment is carried out with $NaBH_4$-inactivated enzyme, no ^3H_2O is found (experiment 2, Table II). The experiments described above establish that 2′dAdo bound to $NaBH_4$-reduced enzyme is converted to adenine. Therefore, adenine formation catalyzed by the $NaBH_4$-reduced enzyme does not involve the release of the C-2′ hydrogen as a proton. Experiments 3 and 4 of Table II show the amount of 2′dAdo bound to the native enzyme and $NaBH_4$-reduced enzyme. The amount of 2′dAdo bound to the native enzyme, and hence the amount of adenine formed, exceeds the amount of ^3H_2O found. This could be due to a tritium-isotope effect. Alternatively, it could be due to the existence of an additional pathway for adenine formation that does not involve abstraction of the C-2′ hydrogen. Data cited in this paper show that such a mechanism exists.

Discussion

The results reported here further confirm the mechanism (eq 2) proposed previously for the inactivation of SAHase by 2′dAdo (Abeles et al., 1980). A central feature of this mechanism is the oxidation of 2′dAdo at C-3′ and the subsequent formation of adenine through a trans-elimination reaction, i.e., the elimination of the C-2′ proton and adenine. We have now confirmed the formation of adenine and, most importantly, have shown that the C-2 proton of 2′dAdo, which is trans to adenine, is released to solvent water. The number of C-2 protons released per enzyme subunit is close to the amount of NADH formed as required by the proposed mechanism. We have obtained no direct experimental evidence for the formation of the keto sugar (eq 2) derived from the deoxyribose moiety of 2′dAdo.

The data show that adenine can be formed from 2′dAdo not only by the elimination mechanism of eq 1 but also by an additional mechanism. Evidence for the additional mechanism is provided by several experiments. The amount of adenine formed from 2′dAdo exceeds the amount of NAD reduced. The interaction of 2′dAdo with SAHase reduced with $NaBH_4$ also results in adenine formation, but the C-2′ hydrogen of 2′dAdo is not released in the solvent. Therefore, adenine can be released from 2′dAdo without NADH formation and without elimination of the C-2′ hydrogen. It is most likely that this mechanism of adenine formation involves enzyme-catalyzed hydrolysis of the glycosidic bond.[3]

Adenine formation with prior oxidation of the ribose moiety of 2′dAdo is closely related to the normal catalytic process. Why does the enzyme also catalyze a hydrolytic cleavage of 2′dAdo? We believe this reflects the manner in which the enzyme interacts with the substrate or the substrate analogue 2′dAdo. Hydrogen-bond formation between SAHase and the adenine moiety of the substrate probably is an important component of the binding interaction between substrate and enzyme. Hydrogen-bond formation is equivalent to protonation of the adenine ring, and protonation of the adenine will facilitate hydrolysis of the glycosidic bond. In addition, it is very likely that binding of the substrate to SAHase results in distortion of the conformation of the ribose moiety. In the course of the catalytic process, C-3′ and C-4′ became sp^2 carbons and must be coplanar so that Michael addition or elimination can occur. This requires considerable distortion of the ribose ring. This distortion can also aid in the hydrolysis of the glycosidic bond of 2′dAdo by facilitating carbonium ion formation and possibly also by straining the glycosidic bond.

The data show that adenine derived from 2′dAdo is tightly bound to the enzyme. This tight binding appears to require the presence of enzyme-bound NADH. When the four NAD molecules of the SAHase tetramer are reduced by $NaBH_4$, the enzyme binds adenine tightly. However, the experiments with 2′dAdo indicate that reduction of two of the four NAD molecules is sufficient to obtain tight binding of adenine to more than two subunits, possibly to all four subunits. Therefore, the presence of NADH on a subunit appears to bring about a conformational change that prevents release of enzyme-bound adenine. The conformational change is transmitted, presumably through intersubunit contact, to the other subunit, which does not contain NADH, so that these subunits also do not release adenine. We suggest that reduction of enzyme-bound NAD converts the enzyme to a "closed" form that does not release bound molecules. This process may be important in the normal catalytic process and prevents escape of the enzyme-bound intermediates. In the normal catalytic process at least two stable intermediates are formed. Release of either of these intermediates would result

[3] The possibility that adenine is formed through a cis elimination cannot be excluded. We consider this possibility very unlikely.

in enzyme inactivation. An analogous observation has been made with yeast aldehyde dehydrogenase. In the presence of a substrate analogue (transition-state analogue?), the enzyme is converted to a closed form, i.e., a form that does not release NAD (Wiseman et al., 1980).

The release of enzyme-bound products derived from 2′dAdo can be accelerated by exogenous 2′dAdo or Ade. When the compounds were added to SAHase–2′dAdo, approximately 50% of the bound radioactivity was released after 15 h. In their absence essentially no radioactive material is released under these conditions. Several mechanisms can be envisioned for this process. Displacement of enzyme-bound compounds, derived from 2′dAdo, may reflect the tight binding of these compounds and the consequent rapid association with the enzyme. In the presence of exogenous substrates, recombination of released compounds with the enzyme is prevented. It is also possible that enzyme-bound compounds are displaced by 2′dAdo in a process analogous to an S_N2 process. Finally, 2′dAdo could bind to one of the unoccupied subunits, which brings about a conformational change, leading to the release of 2′dAdo-derived molecules. It is tempting to speculate that a similar process might occur in the normal catalytic process. Binding of adenosine to these subunits could facilitate product release or possibly some other intermediate step in the catalytic process. Such a process may play a regulatory role.

The composition of the material displaced by 2′dAdo appears to be different from that observed when SAHase–2′dAdo was denatured. Upon denaturation of SAHase–2d′Ado 80–90% of the radioactive material released was adenine. The amount of 2′dAdo was maximally 1.0%, and the material that cochromatographed with 2′-deoxyinosine was maximally 10%. The radioactive material displaced from SAHase–2′dAdo by exogenous 2′dAdo contained a lower percentage of adenine and higher percentage of nucleosides, 2′dAdo and 2′-deoxyinosine. In the experiment in which SAHase–2′dAdo was exposed to unlabeled 2′dAdo, 50% of the radioactive compounds bound to the enzyme were displaced. If the displacement of compounds other than adenine occurred preferentially, one would expect to find a higher percentage of nucleosides in the released material. However, the maximum amount of nucleosides present could not exceed 22%. The actual amount found was in excess of 40%. Preferential displacement of nucleosides can be a contributing factor, but not the entire explanation. Reversible formation of the nucleoside under displacement conditions could provide an additional explanation for the difference in the composition of the compounds observed in the two experiments. It has been proposed that SAHase catalyzes the reversible conversion of Ado to adenine and ribose (Saebo & Ueland, 1979; Ueland & Saebo, 1979).

The data presented here suggest that SAHase consists of two pairs of nonidentical subunits. Only two of the four enzyme-bound NAD molecules are reduced in the presence of Ado or 2′dAdo. Close to two molecules of Ado are bound per tetramer, which do not participate in the catalytic process. We suggest that two of the four subunits do not participate in catalysis but bind Ado as well as 2′dAdo. This raises the interesting question of what function, if any, do the two unreactive subunits serve.

It has been reported that carbocyclic adenosine inhibits SAHase with a K_i of 10^{-9} (Guranowski et al., 1981). We have shown that when carbocyclic adenosine is bound to the enzyme, adenine and NADH are formed. This raises the possibility that formation of adenine occurs through an elimination mechanism, analogous to that which occurs with 2′dAdo. If that is the case, it would be a cis elimination.

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