S-Adenosylhomocysteinase: Mechanism of Reversible and Irreversible Inactivation by ATP, cAMP, and 2′-Deoxyadenosine

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ABSTRACT: Homogeneous *S*-adenosylhomocysteinase (AdoHcyase) from rat liver is a tetrameric enzyme that contains four molecules of tightly bound NAD per mole of enzyme. We report here that incubation of the rat liver enzyme with ATP, Mg^{2+}, and KCl leads to conversion of the active enzyme to an inactive form with release of all enzyme-bound NAD which can be recovered quantitatively by gel filtration. At various concentrations of ATP, the release of NAD corresponds closely with the degree of inactivation, suggesting that the four subunits are equivalent. Hydrolysis of ATP is not required for the inactivation process since nonhydrolyzable ATP analogues can replace ATP in the inactivation process. The ATP-dependent inactivation is fully reversible upon incubation of the inactivated enzyme with NAD. The ATP-dependent inactivation of the enzyme appears to be analogous to the cAMP-dependent inactivation of the enzyme from *Dictyostelium discoideum* described earlier by Hohman et al. (1985) [Hohman, R. J., Guitton, M. C., & Veron, M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4578–4581; Hohman, R. J., Veron, M., & Guitton, M. C. (1985) *Curr. Top. Cell. Regul. 26*, 233–245] but differs from the irreversible inactivation studied earlier by Abeles et al. (1982) [Abeles, R. H., Fish, S., & Lapinskas, B. (1982) *Biochemistry 21*, 5557–5562]. These authors have ascribed the time-dependent inactivation that results from incubation of the enzyme with 2′-deoxyadenosine to the reduction of 2 of the 4 mol of enzyme-bound NAD concomitant with the oxidation of 2′-deoxyadenosine at the C-3′ and concluded that AdoHcyase "probably consists of two nonequivalent pairs of subunits". We have reexamined the mechanism of the inactivation of AdoHcyase by 2′-deoxyadenosine and report here that it consists of the conversion of an active holoenzyme containing 3.6 mol of tightly bound NAD/mol of enzyme to an inactive apoenzyme that may contain from 1.3 to 3.4 mol of enzyme-bound NADH/mol of enzyme and no enzyme-bound NAD.

S-Adenosylhomocysteine (AdoHcy),[1] one of the products of methyl transfer reactions from *S*-adenosylmethionine (AdoMet) and a competitive inhibitor of all such reactions, is metabolized through a single pathway by *S*-adenosylhomocysteinase (AdoHcyase), an enzyme which catalyzes the reversible hydrolysis of AdoHcy to adenosine and L-homocysteine (de la Haba & Cantoni, 1959). The K_m's for AdoMet and the K_i's for AdoHcy of different methyl transferases vary independently and over a 1000-fold range and thereby determine a hierarchy of inhibitions for the various methyl transferases (Cantoni & Chiang, 1980).

The competitive nature of the inhibition by AdoHcy with respect to AdoMet leads to the conclusion that it is the ratio of the intracellular concentrations of AdoMet to AdoHcy rather than their absolute concentrations that determines the degree of inhibition of a given methyl transferase at a given concentration of AdoHcy. It is reasonable to suppose, therefore, that some mechanism must exist for the control of the intracellular activity of AdoHcyase, the only enzyme known to metabolize AdoHcy.

AdoHcyase has been obtained in homogeneous form from various sources: the enzymes from mammalian tissues are tetramers with molecular weights of approximately 190 000 (Richards et al., 1978; Doskeland & Ueland, 1982; Fujioka & Takata, 1981). The plant enzymes are polymers made up of subunits with a molecular weight of 55 000; the enzyme from *Nicotiana tabacum* is a tetramer, M_r 220 000 (Sebestova et al., 1983), whereas the enzyme from lupin seed is a dimer with a molecular weight of 110 000 (Guranowski & Pawelkiewicz, 1977). Finally, a bacterial enzyme, that was crystallized by Shimizu et al. (1984) from *Alcaligenes faecalis*, is composed of six subunits, each with a molecular weight of 48 000.

The mechamism of the enzymatic reaction has been elucidated by Palmer and Abeles (1979), who made the important discovery that each enzyme subunit contains one tightly bound NAD which is used in an internal oxidation–reduction at the 3′-C of the nucleoside substrate. NAD is not released during enzyme catalysis, and addition of NAD during enzyme assay is without effect on enzyme activity. The enzymes from mammalian liver and from *Alcaligenes faecalis* contain, in round numbers, 1 mol of NAD/mol of subunit, but up to 10% of the total NAD may be present in the reduced form (Shimizu et al., 1984; Abeles et al., 1982; Fujioka & Takata, 1981; Richards et al., 1978). The tetrameric enzyme from *Dictyostelium discoideum* appears to be quite different since it contains less than 0.25 mol of NAD and 0.25 mol of NADH/mol of subunit (Hohman et al., 1985a,b). The question of the identity or nonidentity of the four subunits of

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[1] Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; AdoHcyase, *S*-adenosylhomocysteine hydrolase; DZA, 3-deazaadenosine; DZAHcy, *S*-3-deazaadenosylhomocysteine; E_a, active form of AdoHcyase; E_i, inactive form of AdoHcyase; E_{ia}, reactivated form of AdoHcyase; AMPPCP or App$(CH_2)_p$, 5′-adenylyl β,γ-methylenediphosphate; AMPPNP or App$(NH)_p$, 5′-adenylyl β,γ-imidodiphosphate; AMPNPP or Ap$(NH)_{pp}$, 5′-adenylyl α,β-imidodiphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

the tetrameric vertebrate enzymes is not yet fully resolved. Gomi et al. (1985) concluded from analysis of the peptide map obtained after proteolysis of the purified rat liver enzyme that the four subunits are identical, whereas Abeles et al. (1982) deduced from the partial reduction of NAD to NADH during "suicide" inactivation of the enzyme that two of the four subunits may be different and serve a regulatory function. Earlier, Doeskeland and Ueland assigned to the liver enzyme the structure $\alpha_2\beta_2$ (Doskeland & Ueland, 1982). Additional complexity arises from the discovery of Saebo and Ueland (1978), and of Herschfield and Kredich (1978), that a protein found in mouse liver and in human placenta, and capable of binding adenosine and cAMP, but devoid of cAMP-dependent protein kinase activity, is identical with AdoHcyase. It is noteworthy that the cAMP binding activity, which Ueland et al. measured at 4 °C, was very greatly increased after incubation at 37 °C with ATP, Mg^{2+}, and KCl (Ueland & Saebo, 1979). Hohman et al. (1985a,b) have reported that incubation of the enzyme from *Dictyostelium discoideum* with cAMP leads to an analogous loss of activity which could be restored by incubation with NAD. Here we present data showing that incubation of the rat liver AdoHcyase with ATP, Mg^{2+}, and KCl leads to conversion of the active enzyme (E_a) to an inactive form (E_i) with release of all the enzyme-bound NAD. Almost complete recovery of enzyme activity can be achieved by incubation of E_i with NAD, a procedure which converts E_i to an enzyme form that binds NAD tightly (E_{ia}). E_a does not bind cAMP; by contrast, as would be anticipated, E_i binds cAMP, but interaction with this ligand does not restore AdoHcyase activity. We will report elsewhere data that indicate that the conformation and some properties of E_a, E_i, and E_{ia} are different.

Materials and Methods

Materials. Frozen male Wistar rat livers were from Pel-Freeze (Rogers, AR); calf intestine adenosine deaminase (type IV), alcohol dehydrogenase, thiazolyl blue, and 5-ethylphenazinium ethyl sulfate (PES) were from Sigma; Na-Bicine was from Calbiochem. 3-Deazaadenosine was obtained from the Southern Research Institute (Birmingham, AL); Ag-adenosine type 3 was from Pharmacia P-L.; App(CH_2)p (5′-adenylyl β,γ-methylenediphosphate), App(NH)$_p$ (5′-adenylyl β,γ-imidodiphosphate), Ap(NH)pp (5′-adenylyl α,β-imidodiphosphate), and ATPδS [adenosine 5′-*O*-(3-thiotriphosphate)] were purchased from Sigma. Orthovanadate was purchased from Fisher Scientific Co. [^{14}C]AdoHcy was synthesized enzymatically and purified on a Vydac column (Hoffman, 1975).

NAD and NADH Determination. NAD and NADH were measured by a cycling assay according to the procedure of Bernofsky and Swan (1973) that uses alcohol dehydrogenase and thiazolyl blue as the terminal electron acceptor. To measure independently (1) the total nicotinamide adenine diphosphate content (NAD + NADH), (2) the total NAD content, and (3) the amount of NAD + NADH not enzyme bound, the enzyme samples were prepared as follows: for the estimation of the total NAD + NADH, the enzyme sample was denatured by addition of 2 volumes of ethyl alcohol, dried under a stream of nitrogen, and redissolved in water. For the independent measurement of total NAD, the sample was precipitated with 1 volume of 7 N perchloric acid to destroy the reduced form of the nucleotide. After centrifugation, 1 volume of 0.12 M Bicine buffer, pH 7.0, was added to the supernatant fluid which was then carefully neutralized by addition of 5 N KOH. It is important to avoid pHs higher than 7.0–7.5 since NAD is unstable in alkali; the NADH content was then calculated by subtraction of the NAD value from the total nucleotide content. Since enzyme-bound NAD is not accessible to alcohol dehydrogenase in the cycling assay, the amount of nicotinamide adenine diphosphates not enzyme bound can be estimated directly, without enzyme denaturation. Since some enzyme samples contained DTT, appropriate controls were performed to eliminate interference by DTT in the cycling assay.

Enzyme Activity Assays. In the synthetic direction, enzyme activity was assayed by measuring the synthesis of *S*-3-deazaadenosylhomocysteine (DZAHcy) in a 200-μL reaction mixture consisting of 0.1 mM 3-deazaadenosine (DZA), 10 mM DL-homocysteine, 1 mg·mL^{-1} bovine serum albumin, and 10 mM Hepes buffer, pH 7.0. The reaction was started by the addition of the enzyme and stopped after 10-min incubation at 37 °C by adding 20 μL of 3 M perchloric acid. The protein precipitate was removed by centrifugation, the sample was neutralized with 40 μL of 1 M K_2CO_3, and the precipitated potassium perchlorate was removed by centrifugation. DZA and DZAHcy were analyzed by HPLC on a Altex Ultrasphere C 18 ODS reverse-phase column (4.6 × 25 cm) equilibrated with 0.1 M KH_2PO_4, pH 4.3, containing 0.5 M NaCl and 5.5% methanol and operated with a flow rate of 1.3 mL·min^{-1}. Elution times for DZA and DZAHcy were 7.9 and 9.2 min, respectively.

The reaction mixture used for assay in the hydrolytic direction contained, in a final volume of 200 μL, 0.125 mM [^{14}C]AdoHcy, 10 mM Hepes, pH 7.0, 1 mg·mL^{-1} bovine serum albumin, and 4.4 IU of adenosine deaminase. The reaction was stopped after a 10-min incubation at 37 °C by the addition of 20 μL of 5 M formic acid. Reaction products were analyzed as described (Kim et al., 1983).

AdoHcyase Purification. Frozen male Wistar rat livers were homogenized in 2 volumes (w/v) of 10 mM acetic acid, 1 mM DTT, and 1 mM EDTA and centrifuged 15 min at 28000*g*, and the supernatant fluid was filtered through glass wool. All subsequent steps were performed as described by Kajander and Raina (1981). Agadenosine type 3 (agarose–hexane–adenosine) was used for the affinity chromatography procedure.

Protein Concentration. AdoHcyase concentration was measured at 280 nm by assuming an absorption coefficient of 13.0 for a 1% solution (Ueland et al., 1978). Enzyme concentrations of diluted samples were measured by the method of Bradford using Bio-Rad reagents. The Bradford method overestimates the protein content by a factor which varied, as determined experimentally, between 1.7 and 1.9 for different enzyme preparations.

Gel Filtration on the Sephadex G-50 Column (G-50 Procedure). Gel filtration was used for separation of the enzyme from free ligands and other small molecules during inactivation and reactivation procedures. Columns were prepared from Falcon 2-mL plastic serological pipets with the tip filled with glass wool. Columns (4 × 130 mm) were filled with 1.8 mL of Sephadex G-50 equilibrated with an elution buffer composed of 15 mM Hepes, pH 7.0, containing 0.6% polyethylene glycol (M_r 6000) and in some cases 1 mM DTT. After application of the sample (maximum volume 150 μL), three-drop fractions were collected. Protein was found in fractions 7–9, and elution of NAD began in fraction 10.

Binding of Radioactive Ligands. Gel filtration on Sephadex G-50 columns was used to determine the binding of [^{14}C]NAD or [^{3}H]cAMP to the enzyme; radioactive ligands were added to the enzyme solution as described in the text followed by determination of the micromoles of radioactive ligand that

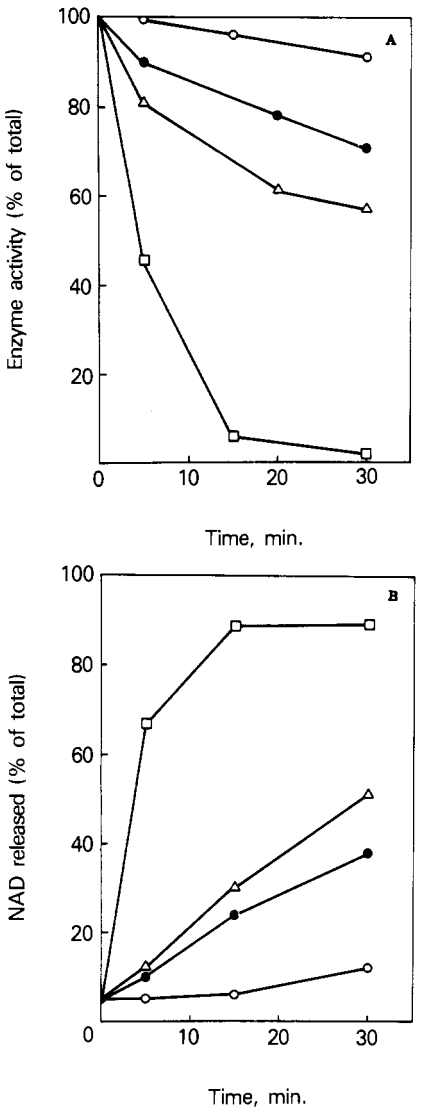

FIGURE 1: (A) Inactivation of AdoHcyase by ATP. (B) Release of NAD during ATP inactivation. Eighteen micrograms of E_a was incubated at 37 °C in 15 mM Hepes, pH 7.0, containing 150 mM KCl, 8 mM magnesium acetate, and 0.05 mM DTT in the presence of 0 (○), 2 (●), 3 (△), and 8 (□) mM ATP, respectively. At times indicated, 30-μL aliquots were taken to assay the NAD release by the cycling assay, and 10-μL aliquots were diluted for the activity measurements. Activity (A) is expressed as the percent of the initial value; NAD release (B) is shown as the percent of the total NAD detected as "free" NAD.

remained bound to the protein fractions after gel filtration.

RESULTS

Inactivation of AdoHcyase upon Incubation with ATP. As shown in Figure 1A, incubation at 37 °C with 8 mM ATP, Mg^{2+}, and KCl results in almost complete inactivation of AdoHcyase. The degree and rate of inactivation depend on the concentration of ATP (Figure 1A). With 8 mM ATP, inactivation is essentially complete within 15 min; with 3 mM ATP, the rate of inactivation is slower, and 30 min is required for 50% inactivation. Table I shows that Mg^{2+}, ATP, and KCl are required for the inactivation process. In the absence of either Mg^{2+} or ATP, little or no inactivation is observed; in the absence of KCl, only partial (68%) inactivation is seen. NaCl (not shown) was not as effective as KCl, in agreement with earlier findings by Ueland (1982), who reported that NaCl was not as effective in the inactivation of the mouse liver enzyme induced by incubation with ATP, cAMP, and other nucleotides. The data in Figure 1 and Table I are from experiments in which the enzyme activity was measured in the direction of hydrolysis. Analogous results were obtained when the enzyme was assayed in the direction of synthesis.

Table I: Requirements for Inactivation of AdoHcyase[a]

preincubation in presence of	AdoHcy hydrolyzed (μmol)	act. (% of control)
(1) no addition	5.4	100
(2) ATP, KCl, Mg^{2+}	0.36	7
(3) KCl	5.74	106
(4) KCl, Mg^{2+}	5.92	110
(5) ATP, KCl	5.32	98
(6) ATP, Mg^{2+}	1.72	32

[a] Nine micrograms of enzyme protein was preincubated for 50 min at 30 °C[3] (except for condition 6 which was incubated for 70 min) in a final volume of 120 μL containing 15 mM Hepes, pH 7.0, 1 mM DTT, and, where indicated, the following compounds: 150 mM KCl, 8.3 mM magnesium acetate, and 8.3 mM ATP. After preincubation, 40 μL of each reaction mixture was assayed in the direction of hydrolysis as described under Materials and Methods.

Table II: Distribution of NAD in G-50 Fractions[a]

enzyme	enzyme-bound NAD (in protein fraction) (% total NAD)	nonbound NAD (free nucleotides) (%)
E_a	92.5	7.5
E_i	1.5	98.5

[a] Enzymes were passed through the G-50 column as described under Materials and Methods, and nucleotides were assayed in the protein fraction and in the nucleotide fraction. E_a, active enzyme containing 3.7 mol of NAD/mol of enzyme; E_i, enzyme after inactivation in the presence of 8 mM ATP. Distribution is expressed as percent of total nucleotides.

AdoHcyase Inactivation Is Due to Dissociation of Enzyme-Bound NAD. In an attempt to explain the mechanism of the inactivation obtained by incubation with ATP, Mg^{2+}, and KCl, we measured the NAD content of the enzyme before and after inactivation. Different preparations of active AdoHcyase from rat liver (E_a) were found to contain between 0.83 and 0.92 mol of NAD and 0–0.1 mol of NADH/mol of subunit, in good agreement with earlier data of Abeles et al. (1982) and of Richards et al. (1978) for the beef liver enzyme. Shimizu et al. (1984) reported that the bacterial enzyme contains 0.85 mol of NAD and 0.1 mol of NADH/subunit. After inactivation with 8 mM ATP, Mg^{2+}, and KCl, the inactivated enzyme (E_i) contains 0.075 mol of NAD/mol of enzyme subunit and retains 10% of the initial activity (Table I). Complete inactivation was never observed. The fraction of NAD released from the enzyme can be separated by gel filtration from the protein-bound fraction and quantitatively recovered in the G-50 eluates (Table II). These observations differ from earlier reports of Ueland (1982), who could not detect dissociation of NAD from mouse liver enzyme upon incubation with ATP, Mg^{2+}, and KCl.

The kinetics of NAD release as a function of the concentration of ATP are shown in Figure 1B. It can be seen that the NAD release corresponds closely with the degree of inactivation, a result that indicates that the 4 mol of NAD bound to the enzyme is equivalent with respect to ATP-dependent dissociation from the enzyme. The release of NAD has been measured only in the presence of ATP, KCl, and Mg^{2+}. In the absence of KCl, as shown in Table I, a partial inactivation is observed, but we have not determined if NAD is released also in this case.

Reactivation of E_i by NAD. The experiments described in Figure 1 showing that the course of NAD release corresponds quantitatively with the extent of enzyme inactivation suggested, by analogy with the findings of Hohman et al. (1984), that

Table III: Activity and NAD Content of E_a, E_i, and E_{ia}[a]

enzyme	enzyme concn (nmol/mL)	mol of NAD/mol of enzyme[d]	NAD content[f] (%)	sp act. (EU/mL)[e]	act. (%)
E_a	9.0	3.77[b]	100	5.4	100
E_i	2.58	0.3[b]	8	0.56	10
E_{ia}	0.76	2.9[c]	81	5.1	94

[a] 270 μg of E_a was incubated in the presence of 15 mM Hepes, pH 7.0, 1 mM DTT, 150 mM KCl, 8 mM magnesium acetate, and 8 mM ATP in a final volume of 150 mL for 30 min at 37 °C and then passed through a G-50 column at 4 °C as described under Materials and Methods. Fractions with E_i were pooled, and enzyme activity and NAD content were determined. 59 μg of E_i was incubated in the presence of 0.08 mM [^{14}C]NAD for 30 min at 37 °C. Labeled enzyme (E_{ia}) was separated from nonbound [^{14}C]NAD by gel filtration on a G-50 column. E_{ia} was assayed for enzyme activity, protein concentration, and radioactivity. [b] NAD estimated by the cycling assay, using perchloric acid or ethanol for enzyme denaturation; see Materials and Methods. [c] NAD analysis is based on [^{14}C]NAD binding. [d] Protein estimated by the Bradford method and corrected as described under Materials and Methods. [e] 1 EU corresponds to 1 μmol of DZAHcy synthesized in 1 min. [f] Calculated by assuming a molecular weight of 190 000.

E_i might be reactivated upon incubation with NAD. This in fact is the case as documented in Table III. The reactivation is time dependent (not shown) and is completed after 15 min at 30 °C. In the reactivation reaction, the K_m for NAD is about 15 μM, and Mg^{2+} ions are not required. Neither NADH nor NADP can replace NAD for the reactivation of E_i (data not shown). In a typical experiment, shown in Table III, E_i retained 10% of the activity and 8% of the enzyme-bound NAD; after reactivation, E_{ia} regained 94% of the activity and had 81% of the NAD content of E_a. It may be noted that upon incubation of E_a with NAD the amount of enzyme-bound NAD increased from 3.7 to 4.0 mol/mol of enzyme and there was a small but reproducible increase in enzyme activity (data not shown). Hohman et al. (1984) also reported that incubation of the *Dyctyostelium* enzyme with NAD resulted in an increase in enzyme activity.

What Is the Function of ATP in the Inactivation Process? Incubation with nonhydrolyzable analogues of ATP such as ATPγS, AMPPCP, AMPPNP, and AMPNPP is almost as effective as incubation with ATP. Thirty minutes incubation with these analogues at 8 mM concentrations, in the presence of Mg^{2+} and KCl, resulted in 90, 67, 44, and 55% inactivation, respectively. These results indicate that hydrolysis of ATP is not required for the inactivation process. AdoHcyase exhibits facile interaction with many different nucleosides, and therefore, the possibility that the effects of ATP might be due to the presence of an impurity had to be considered. While it is virtually impossible to rule out the possibility that the effects of ATP might be due to the presence of an unknown impurity, it has been determined by HPLC analysis that no UV-absorbing impurities were present within the detection limits of the method (less than 1 part/1000). It is of interest to point out that the requirement of ATP, Mg^{2+}, and KCl for inactivation of AdoHcyase resembles those reported for the inhibition of the (Na,K)-ATPase by a factor present in ATP preparations from muscle and identified as Na_3VO_4 by Cantley et al. (1977). We therefore tested orthovanadate in the presence and the absence of ATP, Mg^{2+}, and KCl for its ability to inactivate AdoHcyase but could not find any effect. The ability of synthetic ATP analogues to replace ATP in the inactivation process is further evidence against the idea that the inactivation may be due to an impurity.

Does cAMP Play a Role in the Inactivation Process of Rat Liver AdoHcyase? A possible role of cAMP in the inactivation of rat liver AdoHcyase was investigated in detail for several reasons. In the first place, Ueland and Doeskeland (1978) had reported that incubation at 37 °C with cAMP would increase the ability of AdoHcyase to bind cAMP by a process analogous to the activation of cAMP binding seen upon incubation with ATP. Furthermore, while this work was in progress, Hohman et al. (1984) reported that incubation of AdoHcyase from *Dictyostelium* with 100 μM cAMP and 20 mM NaCl at pH 6.5 resulted in a slow (18 h) but essentially complete inactivation of the catalytic activity. The inactivated *Dictyostelium* enzyme could be reactivated by NAD (Hohman & Veron, 1984) in a manner quite analogous to that which we have described above for the rat liver enzyme, after incubation with ATP. We found that the rat liver enzyme can be partially (25–50%) inactivated after 30-min incubation with 1–2 mM cAMP in the presence of Mg^{2+} and KCl.

Rat liver AdoHcyase does not bind appreciable amounts of [^{3}H]cAMP or [^{14}C]NAD. On the other hand, after ATP, Mg^{2+}, and KCl inactivation, E_i binds about 3 mol of cAMP–mol of enzyme. This observation confirms Ueland's earlier findings (Ueland, 1982) that the interaction of E_i with cAMP does not restore enzymatic activity. Upon incubation of [^{3}H]cAMP-labeled enzyme with NAD, all the cAMP is displaced, and the enzyme binds NAD with recovery of enzymatic activity. One explanation of these data is that cAMP and NAD compete for the same binding site on the enzyme, but further work will be needed to determine if this explanation is valid and what its physiological significance may be, if any.

Mechanism of AdoHcyase Inactivation by 2′-Deoxyadenosine. As first shown by Hershfield (1979), incubation of AdoHcyase from human placenta with adenosine, 2′-deoxyadenosine, and other adenosine analogues results, in the absence of homocysteine, in a time-dependent or "suicide" inactivation of the enzyme. The mechanism of this type of inactivation was investigated by Abeles et al. (1982), who showed that 2′-deoxyadenosine reduces enzyme-bound NAD to NADH with the concomitant oxidation of 2′-deoxyadenosine to its 3′-keto derivative; the 3′-keto analogue spontaneously eliminates adenine, and the enzyme-bound NADH cannot be reoxidized. Abeles et al. (1982) reported that upon incubation with 2′-deoxyadenosine *complete* inactivation of the beef liver enzyme resulted in the reduction of *only two* of the four NADs that are bound to the enzyme and on the basis of these observations suggested that AdoHcyase consists of two unequal pairs of subunits one of which participates in enzyme catalysis while the other has an as yet undefined regulatory function (Abeles et al., 1982). It was of interest, therefore, to investigate in more detail the effect of incubation of the homogeneous rat liver enzyme with 2′-deoxyadenosine especially in light of the results of Gomi et al. (1985), who, from analysis of peptides obtained by partial fragmentation of the enzyme, concluded that AdoHcyase consists of four identical subunits. The results of our experiments are summarized in Table IV.

It should be noted first of all that, in agreement with Abeles et al. (1982), we consistently observed that more than 95% of the enzyme activity was lost after 30-min incubation with 2 mM 2′-deoxyadenosine. However, the amount of enzyme-bound NADH formed was found to vary between 30% and 85% of the total nucleotide content depending on the experimental conditions, the amount of NADH formed being smaller when the reaction mixture did not contain glycerol, greater when glycerol was added to the inactivation mixture (Table IV, experiments D and F), and greatest when Mg^{2+} and KCl were also added. It must be pointed out that the

Table IV: Inactivation of AdoHcyase by 2′-Deoxyadenosine[a]

expt[b]	enzyme (μg/μL)	incubn time (min)	additions	act. (%)	nucleotide: enzyme-bound NAD (%)	nucleotide: enzyme-bound NADH (%)	nucleotide: free NAD (%)	nucleotide: free NADH (%)
A	0.40	0		100	90	10	5	0
B	0.40	30		91	90	10	7	0
C	0.46	30	2 mM dAdo	4	68	32	68	8
D	0.40	30	2 mM dAdo	4	32	68	32	16
E	0.43	30	2 mM dAdo	3.5	15	85	15	20
F	0.29	30	2 mM dAdo	3	28	72	28	0

[a] In each experiment, aliquots for enzymatic activity measurements and for the NAD cycling assay were taken from the same inactivation mixture at the times indicated. All the NAD and NADH measurements were done as described under Materials and Methods and expressed as the percent of the total NAD + NADH content. NADH values were obtained by subtracting NAD values from 100. Activity is calculated as the percent of the initial value (measured in the direction of synthesis). [b] Experiments were as follows: A and B, control at time 0 and after 30 min at 37 °C; C and F, the enzyme before the inactivation was passed through a G-50 gel filtration column in order to remove glycerol and diulted in 20 mM Hepes buffer, pH 7.0; D and E, the enzyme was not passed through the G-50 column; the concentration of glycerol in the inactivation mixture (20 mM Hepes, pH 7.0) was 3% or less; E, KCl and magnesium acetate were added to a final concentration of 150 and 8 mM, respectively; F, glycerol was added to the inactivation mixture (20% of the total volume).

cycling assay measures total free nucleotides (NAD + NADH); in other words, enzyme-bound NAD and/or NADH can be determined only after enzyme denaturation. Measurement of NAD + NADH before and after enzyme denaturation yielded valuable information and allowed us to resolve the apparent disagreement between the extent of enzyme inactivation and NADH formation that results from treatment with 2-deoxyadenosine. It can be seen in experiments A and B of Table IV that in the fully active enzyme 95% of the total nicotinamide nucleotides is enzyme bound and as such not available to the cycling assay unless the enzyme is denatured and furthermore that the 5% that was "free" was in the oxidized form. After treatment with 2′-deoxyadenosine in the absence of glycerol (Table IV, experiment C), 68% of the total nucleotides remained in the oxidized form, and all of it was measurable as "free NAD"; 32% was reduced to NADH (75% of the NADH was enzyme bound, and 25% was free); similarly (experiment D), after incubation in the presence of glycerol, 32% of the total nucleotides remained in the oxidized form, all of which was free; 68% of the total was reduced (70% of the NADH was enzyme bound, and 30% was free). The results for experiments E and F are also shown in Table IV. As a further verification of the results shown in Table IV, we measured the nucleotide content of the ultrafiltrate obtained by passage of the reaction mixtures of the 2′-deoxyadenosine experiments through the Amicon Centrifree micropartition system. We found (not shown) that all the NAD remaining in the oxidized form after treatment with 2-deoxyadenosine could be recovered in the ultrafiltrate. Most of the NADH remained enzyme bound, but a small fraction was found in the ultrafiltrate; the total amount of nucleotides recovered in the ultrafiltrates was the same as the amounts of nucleotides (NAD + NADH) measured as free in the reaction mixtures of the 2′-deoxyadenosine experiments.

The results described above confirm and extend the findings of Abeles et al. and modify the interpretation of these authors; the complete inactivation of AdoHcyase after incubation with 2′-deoxyadenosine is due to the combination of two events: (a) reduction of a fraction of the enzyme-bound NAD and (b) release into the supernatant fluid of the fraction that is not reduced. The extent of NAD reduction is variable and depends on the experimental conditions.

We conclude, therefore, that in the absence of L-homocysteine, the mechanism of inactivation of the enzyme upon incubation with 2-deoxyadenosine, and presumably other oxidazable adenosine analogues, consists in the conversion of the homoenzyme containing 4 mol of NAD/mol of tetramer to an apoenzyme that contains variable amounts of NADH and no NAD.[2]

Incubation of the 2′-deoxyadenosine-inactivated enzyme with NAD, with or without pretreatment with ATP, Mg^{2+}, and KCl, resulted in a partial reactivation (20–30% of initial activity). This result fits well with the mechanism outlined above for the inactivation by 2′-deoxyadenosine: only a fraction of the NAD binding sites in the enzyme becomes accessible to exogenous NAD as a result of suicide inactivation, and it is easy to visualize that only these sites could bind NAD, upon incubation with the nucleotide. Our data do not support the hypothesis that AdoHcyase is made up of two pairs of subunits having different functions.

Discussion

The observations reported here confirm and extend earlier observations of Ueland (1982) and of Hohman et al. (1985a,b). A key feature of the results reported here and of those reported earlier by Hohman et al. (1985a,b) is the demonstration that the tightly bound NAD, that is essential for enzymatic catalysis, can be made to dissociate reversibly from the enzyme under certain conditions. While at first glance these results seem to provide a clue regarding a regulatory mechanism of the activity of AdoHcyase, the problem is complex, and the nature of a regulatory mechanism is not yet clear. There are a number of interesting differences in the interaction between NAD and the enzymes from rat liver and from *Dictyostelium* that suggest that the enzyme-bound NAD dissociates more easily from the *Dictyostelium* enzyme than from the rat liver enzyme. In the first place, while the rat liver enzyme contains 3.6–4.0 mol of NAD per tetramer, and 0–0.4 mol of NADH, the *Dictyostelium* enzyme contains only 0.86 mol of NAD and 0.86 mol of NADH per tetramer (Hohman et al., 1985a,b). Moreover, as reported by Hohman et al. (1985a,b), NAD is needed during the early stages of enzyme purification to stabilize the enzyme, whereas NAD has been not found to be needed during the purification of the enzyme from rat, hamster (Kim et al., 1983), or beef liver. Unlike the rat liver enzyme, the four subunits of the *Dictyostelium* enzyme undergo dissociation upon gel electrophoresis under nondenaturing con-

[2] The irreversible inactivation seen after incubation with 2′-deoxyadenosine has been observed in vivo (Hershfield et al., 1979).

[3] The initial inactivation experiments were carried out at 30 °C because that was the temperature that was used by Ueland and Doskeland (1978) in the cAMP binding experiments. Later experiments on inactivation and reactivation were carried out at 37 °C, and analogous results at the two temperatures were observed.

ditions unless NAD is present during electrophoresis, an observation which suggests that the quaternary structure of the enzyme is stabilized by NAD. Removal of NAD from the *Dictyostelium* enzyme requires milder treatment than removal of NAD from the rat liver enzyme: an accurate comparison cannot be made, however, because the experimental conditions are quite different; complete removal of NAD from the rat liver enzyme requires 8 mM ATP, Mg^{2+}, and KCl, and 50% release can be observed with 2–3 mM ATP in the presence of Mg^{2+} and KCl. With the *Dictyostelium* enzyme, 75% of the enzyme-bound NAD is released upon incubation with 100 μM cAMP in the absence of Mg^{2+} ions or KCl.

What the physiological significance is, if any, of the ATP or cAMP inactivation described here and in earlier reports is not clear. The concentration of ATP required for 50% inactivation (2–3 mM) is of the same order of magnitude as the physiological concentration (1–2 mM). It is conceivable that uneven intracellular distribution of ATP might result in higher localized concentrations. These considerations are not valid for cAMP, since the intracellular concentration of this nucleotide is supposed to be in the micromolar range or at least 2 orders of magnitude lower than that needed for in vitro inactivation of the hydrolase. Unpublished experiments in this laboratory using RAW 264 cells or C6 cells in culture have shown that a 200–300-fold increase in intracellular cAMP concentration, whether brought about by forskolin, cholera toxin, or isoproterenol and 3-isobutyl-1-methylxanthine (IBMX), does not result in increases in intracellular AdoHcy concentration or in a decrease in the activity of AdoHcyase (R. P. Aksamit, unpublished results). Since increases in intracellular AdoHcy concentration are easily detectable when AdoHcyase is inhibited by any one of a variety of agents, we conclude that even large variations in intracellular concentrations of cAMP do not result in inhibition of AdoHcyase. Further work will be required before the complexity of the regulatory controls of this interesting enzyme is fully understood.

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Registry No. NAD, 53-84-9; ATP, 56-65-5; cAMP, 60-92-4; 2′-deoxyadenosine, 958-09-8; *S*-adenosylhomocysteinase, 9025-54-1.

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