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S-Adenosylhomocysteine hydrolase from human placenta

Affinity purification and characterization

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S-Adenosylhomocysteine hydrolase (EC 3.3.1.1) was purified to homogeneity from human placenta by using *S*-adenosylhomocysteine–agarose affinity chromatography. The enzyme is a tetramer with a native M_r of 189000 and subunit M_r of 47000–48000; there were nine cysteine residues per subunit and no disulphide bonds. The pI was 5.7. H.p.l.c. analysis revealed that the enzyme contained four molecules of tightly bound cofactor (NAD) per tetramer, of which 10–50% was in the reduced form. The enzyme had four binding sites per tetramer for adenosine, of which 10–35% were found to be occupied. Two types of adenosine-binding sites could be distinguished on the basis of differences in rates of dissociation of the enzyme–adenosine complex, and by examining binding of adenosine at 0°C and 37°C. The enzyme catalysed the interconversion of adenosine and 4′,5′-dehydroadenosine; the equilibrium constant for this reaction was 2.1 and favoured 4′,5′-dehydroadenosine formation. Variability in the specific activity of preparations of *S*-adenosylhomocysteine hydrolase was related to the NAD^+/NADH ratio of the preparation. The capacity to bind radioactively labelled adenosine depended on the adenosine content of the purified enzyme. The rate of adenosine binding and the sensitivity of *S*-adenosylhomocysteine hydrolase to inactivation by adenosine were both diminished in the absence of dithiothreitol.

S-Adenosylhomocysteine hydrolase (AdoHcyase, EC 3.3.1.1) catalyses the reversible hydrolysis of AdoHcy, a product and competitive inhibitor of *S*-adenosylmethionine-dependent transmethylation reactions, to adenosine and L-homocysteine (de la Haba & Cantoni, 1959). In addition to its enzymic activity, we have shown that AdoHcyase can form a stable complex with adenosine and is the major high-affinity adenosine-binding protein in cytoplasm (Hershfield & Kredich, 1978). The physiological function of adenosine binding is unknown, but it has been postulated that AdoHcyase may sequester intracellular adenosine and in some manner mediate the effects of this nucleoside (Ueland, 1982*b*; Ueland & Helland, 1983).

Because it is thermodynamically unfavourable (de la Haba & Cantoni, 1959), AdoHcy hydrolysis depends on efficient metabolism of adenosine and homocysteine, as well as on adequate AdoHcyase activity. Impaired elimination of adenosine may lead to elevated concentrations of AdoHcy and inhibition of methylation when the enzyme adenosine deaminase (ADA, EC 3.5.4.4) is genetically absent or pharmacologically inhibited (Kredich & Martin, 1977; Kredich & Hershfield, 1979). A decrease in the absolute activity of AdoHcyase may also limit AdoHcy catabolism in ADA

Abbreviations used: AdoHcy, *S*-adenosylhomocysteine; AdoHcyase, *S*-adenosylhomocysteine hydrolase; ADA, adenosine deaminase.

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deficiency. Thus 2′-deoxyadenosine, which accumulates in ADA deficiency, as well as the antiviral agent adenine arabinoside, causes the irreversible inactivation of AdoHcyase (Hershfield, 1979; Hershfield & Kredich, 1980). AdoHcyase inactivation occurs in the erythrocytes of ADA-deficient children (Hershfield *et al.*, 1979*a*), and AdoHcy accumulation to concentrations that interfere with some nucleic acid methylation reactions has been observed in the lymphoid cells of patients undergoing treatment with an inhibitor of ADA, 2′-deoxycoformycin, given alone and in combination with adenine arabinoside (Hershfield *et al.*, 1983, 1984). We have mapped the structural gene for human AdoHcyase to the long arm of chromosome 20 (Hershfield & Francke, 1982; Mohandas *et al.*, 1984), which is the location for the gene for ADA (Tischfield *et al.*, 1974; Philip *et al.*, 1980). This finding has raised the possibility of an evolutionary as well as a functional relationship between AdoHcyase and ADA.

In our previous studies of the effects of ADA deficiency on AdoHcyase activity, and in continuing studies of the possible relatedness of ADA and AdoHcyase, we have worked extensively with AdoHcyase from human placenta. In the present paper we describe an affinity purification scheme for this enzyme, some of its physical and catalytic properites, and factors that influence its adenosine-binding characteristics.

Materials and methods

Materials

[8-^{14}C]Adenosine (56–59Ci/mol) and [2-^{3}H]-adenosine (18Ci/mmol) were purchased from Moravek Biochemicals; [*carbonyl*-^{14}C]nicotinamide (53Ci/mol) and [8-^{3}H]adenine (23Ci/mmol) were purchased from Amersham International. Ultrogel AcA-34 was purchased from LKB, CM-Sephadex (C-50) was from Pharmacia, DEAE-cellulose preparations DE-52 and Cellex D were from Whatman and Bio-Rad Laboratories respectively, and *S*-adenosylhomocysteine–agarose was from Bethesda Research Laboratories. Hydroxyapatite (Biogel HT) was from Bio-Rad Laboratories. The ADA inhibitors 9-*erythro*-(2-hydroxynon-3-yl)adenine and 2′-deoxycoformycin were obtained from Wellcome Research Laboratories and Warner–Lambert/Parke–Davis respectively. Non-radioactive nucleosides were obtained from Sigma Chemical Co. or from PL Biochemicals. 4′,5′-Dehydroadenosine was obtained from PL Biochemicals. The authenticity of this compound was documented by n.m.r. and mass spectroscopy. The radiochemical purity of all radioactive compounds was checked by h.p.l.c., and where necessary compounds were repurified.

Methods

Assay of AdoHcyase activity. AdoHcyase activity was determined by measuring the synthesis of [^{14}C]AdoHcy from [8-^{14}C]adenosine and homocysteine (Hershfield, 1979). AdoHcy hydrolysis was assayed by using a coupled spectrophotometric procedure. Assay mixtures (total volume 0.6ml) contained 10mM-Tris/HCl buffer, pH8.0, 1mM-Na_2EDTA, 2.6 units of ADA (Sigma type 1) and AdoHcyase. The reaction was started by addition of AdoHcy. Inosine formation was monitored at room temperature by following the change in absorbance at 264nm with a Gilford 2600 recording spectrophotometer. Under these conditions the absorption coefficient at 264nm for the conversion of AdoHcy into inosine was $7.76 mM^{-1} \cdot cm^{-1}$. For determination of the K_m for AdoHcy the initial velocity of the reaction was used, since the reaction rate was not linear with time.

Determination of binding of adenosine. Binding of radioactivity labelled adenosine was measured by gel filtration over 0.7cm × 20cm columns of Sephadex G-25 (Hershfield & Kredich, 1978).

Determination of protein. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of M_r. AdoHcyase (80μg) in 0.1ml of 10mM-potassium phosphate buffer, pH7.0, was centrifuged to equilibrium in a double-sector rotor of a Beckman model E analytical ultracentrifuge, and absorbance at 280nm was determined with the use of an automatic scanner. Calculation of M_r assumed a partial specific volume of 0.735ml/g, based on amino acid composition (Cohn & Edsall, 1943). Subunit M_r was determined by sodium dodecyl sulphate/2-mercaptoethanol/polyacrylamide-gel electrophoresis in accordance with the procedure of Laemmli (1970).

Determination of amino acid composition. Enzyme (297μg) was dialysed against 1mM-potassium phosphate, pH7.0, freeze-dried and hydrolysed at 110°C in 6M-HCl in sealed evacuated tubes for 24, 48 and 72h. Hydrolysed samples were analysed on a Beckman amino acid analyser (kindly performed by Dr. T. Vanamann). Half-cystine content was determined after performic acid oxidation and hydrolysis (Hirs, 1967). Tryptophan was determined spectrophotometrically (Edelhoch, 1967). Thiol groups were also determined by measuring the change in absorbance at 412nm during reaction of the sodium dodecyl sulphate-denatured enzyme with 5,5′-dithiobis-(2-nitrobenzoic acid) (Habeeb, 1972).

Determination of pI. pI of the enzyme was determined by the method of Wrigley (1971). After electrophoresis, isoelectric-focusing gels (10cm)

were cut into 0.25cm sections. Each section was eluted with 0.2ml of water for pH determination; then 20μl of 1M-Tris/HCl buffer, pH7.4, containing 10mM-EDTA, 10mM- dithiothreitol and 2mg of bovine serum albumin/ml was added to each fraction, and 25μl was removed for assay of AdoHcyase activity.

Preparation of AdoHcyase containing radioactively labelled NAD+. CEM human T-lymphoblasts (200ml, 2×10^5cells/ml) were grown in Eagle's medium (Gibco) lacking nicotinamide and containing 10% (v/v) dialysed (4 days against 0.9%NaCl) horse serum, and 1μM-[*carbonyl*-^{14}C]nicotinamide (50Ci/mol). Cells were harvested when the cell density reached 10^6/ml. For preparing cells containing [^{3}H]adenine-labelled NAD+, 1litre of CEM (6×10^5cells/ml) was grown for 5h in RPMI 1640 medium (Gibco) containing 10% (v/v) horse serum and 2μM-[8-^{3}H]adenine (145Ci/mol), and then harvested. [^{14}C]Nicotinamide-labelled and [^{3}H]adenine-labelled cell pellets were lysed by three cycles of freezing and thawing in 1ml per 10^9 cells of 10mM-Tris/HCl buffer, pH7.4, containing 1mM-EDTA, followed by centrifugation at $13000 \times g$ for 30min at 4°C. With the [^{14}C]nicotinamide-labelled cells AdoHcyase activity was isolated by gel-filtration chromatography of the extract on a 2.5cm × 95cm column of Ultrogel AcA-34 eluted with 25mM-Tris/HCl buffer, pH7.4, containing 15mM-KCl, 1mM-dithiothreitol and 1mM-EDTA. With the [^{3}H]-adenine-labelled cells the extract was applied to a column (1.5cm × 25cm) of DEAE-cellulose (DE-52) and eluted with a linear gradient of 0–0.2M-KCl in 25mM-Tris/HCl buffer, pH7.4, containing 1mM-EDTA and 1mM-dithiothreitol (total volume 400ml). The AdoHcyase-containing fractions were pooled, concentrated by pressure ultrafiltration, and then chromatographed on Ultragel AcA-34. These purification steps yielded in each case a preparation in which all radioactivity was found in NAD+ (Hershfield *et al.*, 1982). In addition, the radioactivity could be precipitated with mouse monoclonal antibody to purified human placental AdoHcyase, prepared in this laboratory (Hershfield & Francke, 1982).

Determination of AdoHcyase-associated NAD+ by h.p.l.c. NAD+ was released from AdoHcyase (2.5–25μg) by addition of acid to give final concentrations of 0.2M-HCl or 0.5–1M-$HClO_4$ for 0.5–1min on ice, followed by centrifugation for 1min at 4°C in a microcentrifuge. A sample of supernatant was analysed directly in the case of HCl-treated samples; supernatants of $HClO_4$-treated samples were neutralized with a combination of KOH and $KHCO_3$, and then centrifuged briefly to remove precipitated $KClO_4$. Extracted NAD+ was determined routinely by h.p.l.c. on a C18 μBondapak reversed-phase column (Waters) eluted with 50mM-sodium acetate buffer, pH3.9, containing 5% (v/v) methanol at a flow rate of 1.5ml/min. Several other elution systems were equally satisfactory. Analyses were performed with a Waters instrument equipped with a model 6000A pump and a model 440 u.v. detector, the absorbance at both 254 and 280nm being recorded.

Equilibrium for the AdoHcyase-catalysed interconversion of adenosine and 4′,5′-dehydroadenosine. Reaction mixtures (0.1ml) contained 25mM-potassium phosphate buffer, pH7.0, 1μM-2′-deoxycoformycin (to inhibit any trace contamination by ADA activity), AdoHcyase (12.8μg) (omitted from controls) and a total concentration of 100μM of adenosine + 4′,5′-dehydroadenosine prepared by mixing these compounds in adenosine/4′,5′-dehydroadenosine ratios of 100:0, 75:25, 50:50, 25:75 and 0:100. Incubation was carried out at 37°C, and at various times over a 3h period portions were removed and stored at −70°C. After 3h an additional 12.5μg of AdoHcyase was added and the reaction was continued for 2h more to establish that equilibrium had been reached. Adenosine and 4′,5′-dehydroadenosine were quantified by h.p.l.c. on a Partisil 10 SCX cation-exchange column (Whatman) eluted with 40mM-sodium phosphate buffer, pH2.6, at a flow rate of 1.5ml/min. Their elution volumes were 8.3ml and 11.0ml respectively

Purification of placental AdoHcyase

All manipulations were conducted at 4°C.

Preparation of homogenate. A placenta, obtained within 30min of delivery, was minced in an equal volume of 1mM-EDTA/1mM-dithiothreitol containing 10ml of Aprotinin (Sigma Chemical Co.) and 200mg of phenylmethanesulphonyl fluoride (dissolved in 2ml of dimethyl sulphoxide) to inhibit proteolysis, and homogenized in a Waring blender. The homogenate was centrifuged at 10000*g* for 60min.

DEAE-cellulose and CM-Sephadex chromatography. The 10000*g* supernatant was made 75mM with respect to KCl and mixed with an equal volume of DEAE-cellulose (Cellex D) that had been equilibrated with buffer A (25mM-Tris/HCl, pH7.4, containing 1mM-dithiothreitol and 1mM-EDTA) containing 75mM-KCl. The suspension was filtered into a flask containing 200mg of phenylmethanesulphonyl fluoride. The resin was rinsed with about 500ml of equilibration buffer. Solid $(NH_4)_2SO_4$ was added to the combined filtrates with stirring to achieve 70% saturation. After 60min the precipitate was collected by centrifugation (10000*g* for 20min), redissolved in 100–200ml of buffer A, and dialysed for 48h against this buffer. The dialysed material was

loaded on to a column (6cm × 45cm) of DE-52 DEAE-cellulose that had been equilibrated with buffer A. The column was eluted with a linear gradient formed from 1000ml of buffer A containing 25mM-KCl and 1000ml of buffer A containing 175mM-KCl. Fractions containing AdoHcyase activity were pooled, adjusted to pH6.0 with acetic acid, and immediately mixed with an equal volume of settled CM-Sephadex that had been equilibrated with 10mM-Tris/acetate buffer, pH6.0. After 2–3min the slurry was filtered into a flask containing 10ml of 1M-Tris/HCl buffer, pH7.4. The gel was washed with 70ml of 10mM-Tris/acetate buffer, pH6.0, and filtered into the same flask

AdoHcy–agarose affinity chromatography. The enzyme was concentrated by pressure ultrafiltration to a volume of 20–100ml and added to 9ml of AdoHcy–agarose that had been equilibrated with buffer B (50mM-Tris/HCl, pH7.0, containing 1mM-EDTA and 1mM-dithiothreitol). The suspension was gently mixed for 60min at 0°C and then transferred to a 1.5cm-diameter column. Unadsorbed protein was collected over 30–45min, after which the column was washed with 10ml of buffer B containing 0.2M-KCl, followed by 50ml of buffer B containing 0.4M-KCl. AdoHcyase activity was then eluted with 50ml of buffer B containing 0.4M-KCl and 0.2mM-AdoHcy. This fraction was immediately concentrated to about 2ml by pressure ultrafiltration and then dialysed against buffer A containing 20% (v/v) glycerol.

Hydroxyapatite chromatography. The dialysed enzyme was applied to a 2.5cm × 2.5cm column of hydroxyapatite that had been equilibrated with buffer A containing 20% (v/v) glycerol. After a washing with 10–15ml of equilibration buffer, the column was eluted with a linear gradient formed from 50ml of equilibration buffer and 50ml of this buffer containing 125mM-potassium phosphate, pH7.0. Fractions containing a constant ratio of AdoHcyase activity to absorbance at 280nm were pooled, concentrated, by pressure ultrafiltration, and dialysed against buffer A containing 20% (v/v) glycerol. Enzyme was stored at a concentration of 1–2mg/ml at −70°C; less than 10% of activity was lost over 6 months.

Results and discussion

Affinity chromatography of rat liver AdoHcyase has been reported with 6-mercaptopurine riboside–Sepharose (Chabannes *et al.*, 1979) and 8-(3-aminopropylamino)adenosine–Sepharose (Kajander & Raina, 1981). Placental AdoHcyase was not retarded by the latter, or by adenosine linked to epoxy-activated Sepharose, which has been used in the purification of human erythrocyte ADA (Schrader *et al.*, 1976). Briske-Anderson & Duerre (1982) employed a type of AdoHcy–agarose as the final step in the purification of rat liver AdoHcyase. However, it is not clear that this was an affinity resin. There was no demonstration of selectivity for AdoHcyase binding, and elution was achieved simply with low-ionic-strength buffer (1mM-phosphate, pH7.0). In contrast, placental AdoHcyase was not eluted from the AdoHcy–agarose column that we used at 0.4M KCl, but required the presence of AdoHcy in the buffer (Fig. 1). AdoHcy–agarose, eluted with *S*-adenosylmethionine, has been used to purify protein-carboxyl *O*-methyltransferase from calf brain (Kim *et al.*, 1978).

Using the scheme summarized in Table 1 we have purified human placental AdoHcyase 1000–3000-fold to apparent homogeneity with a yield of 20–30%. The affinity-purified enzyme (as well as enzyme obtained by conventional chromatographic procedures) gave a single Coomassie Blue-staining band on sodium dodecyl sulphate/2-mercaptoethanol/polyacrylamide-gel electrophoresis (Fig. 1). Sedimentation equilibrium in the analytical ultracentrifuge yielded a linear plot of the logarithm of protein concentration (absorbance at 280nm) against the square of the distance from the centre of rotation (not shown). A single protein band associated with enzyme activity was obtained after electrophoresis of the undenatured enzyme on 7.5%-polyacrylamide gels

Table 1. *Purification of placental AdoHcyase*

For full experimental details see the text. Enzyme activity was determined in the direction of AdoHcy synthesis. One unit corresponds to the formation of 1μmol of AdoHcy/min.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
1. 10000*g* supernatant	23380	56.7	0.002	100	1.0
2. Cellex D+$(NH_4)_2SO_4$	16720	48.8	0.003	86	1.5
3. DE-52+CM-Sephadex	1300	46.3	0.04	82	20
4. AdoHcy–agarose	10.5	20.2	1.92	36	960
5. Hydroxyapatite	4.4	19.5	4.4	34	2200

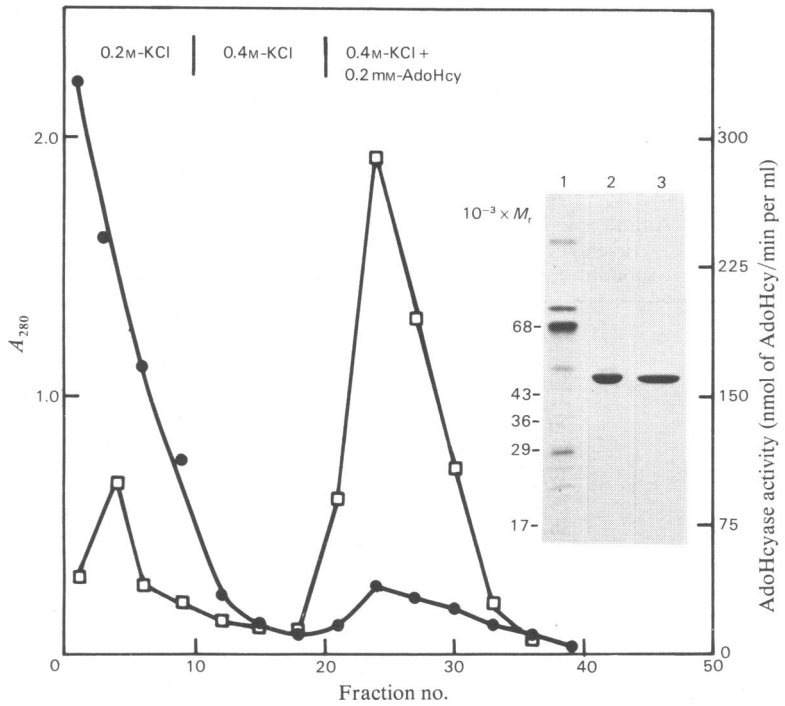

Fig. 1. *Pattern of elution of AdoHcyase activity and protein (A_{280}) from AdoHcy–agarose*

For details of purification and other experimental details see the text. ●, A_{280}; □, AdoHcyase activity. The inset shows Coomassie Blue-stained sodium dodecyl sulphate/2-mercaptoethanol/10%-polyacrylamide-gel analysis of partially purified and purified placental AdoHcyase. Lane 1, sample applied to the AdoHcy–agarose column (17 μg); lane 2, enzyme eluted from the AdoHcy–agarose column with 0.2 mM-AdoHcy/0.4 M-KCl (9 μg); lane 3, purified AdoHcyase after hydroxyapatite chromatography (7 μg).

at pH 8.9, and one protein spot was found on two-dimensional gel electrophoresis by the method of O'Farrell (1975) (not shown).

The M_r of the native enzyme was estimated from analytical ultracentrifugation to be 189000. The subunit M_r estimated from sodium dodecyl sulphate / 2 - mercaptoethanol/polyacrylamide - gel electrophoresis (Fig. 1) was 47000–48000. A subunit M_r of 49000 was calculated assuming nine half-cystine residues per subunit (amino acid determination; Table 2). The absence of disulphide bonds was indicated by the finding of identical mobilities of the bands obtained on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the presence and in the absence of 2-mercaptoethanol, and by the finding of nine reactive thiol groups per subunit upon titration of the sodium dodecyl sulphate-denatured enzyme with 5,5′-dithobis-(2-nitrobenzoic acid) (results not shown). The maximum absorbance of the enzyme was at 277–278 nm, and the $A^{0.1\%}$ was 1.32–1.36, based on protein determined by the method of Lowry *et al.* (1951). The pI of native AdoHcyase was 5.7.

Kinetic characteristics and equilibrium for interconversion of adenosine and 4′,5′-dehydroadenosine

Bovine liver AdoHcyase has been shown to catalyse the conversion of 4′,5′-dehydroadenosine into adenosine, and into AdoHcy in the presence of homocysteine (Palmer & Abeles, 1979). We have confirmed these findings (Fig. 2). The reaction

$$\text{Adenosine} \rightleftharpoons \text{4′,5′-dehydroadenosine} + H_2O$$

was freely reversible and favoured formation of 4′,5′-dehydroadenosine, with $K_{eq.} = 2.1$ (determined as described in the Materials and methods section). K_m values for adenosine, 4′,5′-dehydroadenosine and homocysteine in the AdoHcy synthesis reaction (determined in the presence of saturating amounts of the second substrate) were 0.9, 110 and 200 μM respectively; $V_{max.}$ for AdoHcy synthesis with adenosine as substrate was 4.3–4.5 μmol/min per mg, compared with 0.07 μmol/min per mg with 4′,5′-dehydroadenosine as substrate. The K_m for AdoHcy in the hydrolysis reaction was 7.6 μM.

It seems unlikely that significant formation of free 4′,5′-dehydroadenosine would occur *in vivo*,

Table 2. *Amino acid composition of placental AdoHcyase*
Amino acid analyses were performed on 24h, 48h and 72h hydrolysates. Values for threonine and serine were corrected for decomposition by extrapolation of values obtained from 24h, 48h and 72h hydrolysates. Half-cystine was determined as cysteic acid after performic acid oxidation. Tryptophan was determined spectrophotometrically.

Amino acid	Amino acid composition (average residues/ subunit of M_r 47300)
Lysine	27.2
Histidine	9.9
Arginine	15.5
Aspartic acid/asparagine	41.3
Threonine	20.5
Serine	16.6
Glutamic acid/glutamine	39.5
Proline	18.3
Glycine	36.3
Alanine	36.3
Half-cystine	9.2
Valine	28.9
Methionine	11.3
Isoleucine	19.4
Leucine	36.3
Tyrosine	13.4
Phenylalanine	10.9
Tryptophan	6.8

despite the favourable equilibrium constant, because of the rapid elimination of adenosine by deamination and phosphorylation, although its accumulation might occur in some tissues of ADA-deficient individuals. It is also possible that 4',5'-dehydro derivatives of some adenosine analogues might be formed *in vivo*. This would be most likely in the case of analogues that are substrates for AdoHcyase but poor substrates for adenosine kinase and ADA, e.g. 3-deaza-adenosine (Chiang *et al.*, 1977). The biological effects of 4',5'-dehydro derivatives of adenosine and its analogues are not known.

NAD$^+$, NADH and adenosine content

In previous studies (Palmer & Abeles, 1976; Richards *et al.*, 1978; Fujioka & Takata, 1981; Ueland, 1982*a*) the NAD$^+$ content of AdoHcyase has been measured (after denaturation of the enzyme) by enzymic conversion into NADH, which was then quantified spectrophotometrically. The presence of a peak of weak absorbance in the 320–330nm region of the spectra of AdoHcyases from bovine (Palmer & Abeles, 1979) and rat (Fujioka & Takata, 1981) liver indicates the presence of reduced cofactor on these enzymes, but the amount was not determined. We have used h.p.l.c. analysis to quantify the amounts of NAD$^+$, NADH and

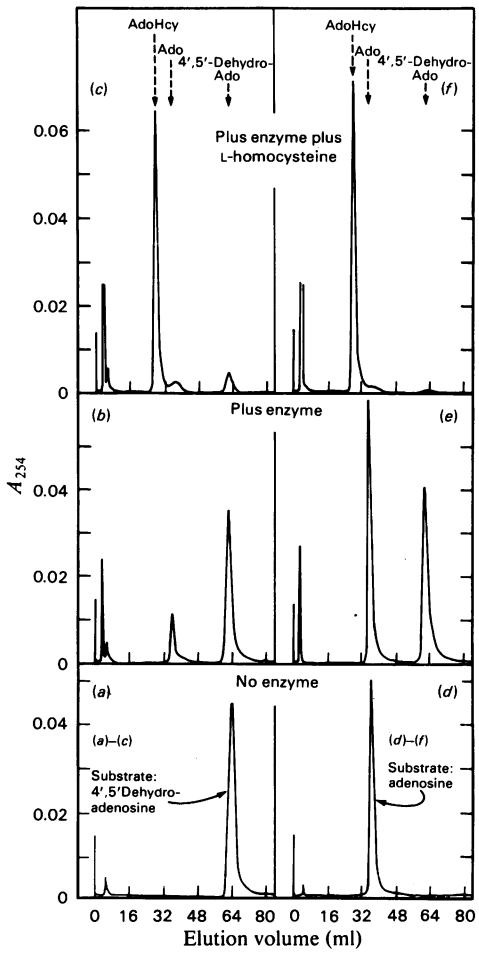

Fig. 2. *AdoHcyase-catalysed conversion of 4',5'-dehydroadenosine into adenosine and AdoHcy*
Reactions were carried out in 10mM-potassium phosphate buffer, pH7.0 (total volume 50μl), containing, as indicated in the Figure, placental AdoHcyase (3.2μg), adenosine (Ado) (0.4mM), 4',5'-dehydroadenosine (0.4mM) and homocysteine (5mM). After incubation at 37°C for 60min, samples of the reaction mixtures were analysed by using a C18 reversed-phase h.p.l.c. column eluted with 50mM-ammonium bicarbonate buffer, pH5.0, containing 2% (v/v) methanol at a flow rate of 2ml/min.

other ligands bound to as little as 1.25μg (about 25pmol of subunit) of placental AdoHcyase (compared with 0.5–5mg required for spectrophotometric methods).

A typical h.p.l.c. tracing (Fig. 3) shows that in addition to NAD$^+$ two other peaks were present in cold acid extracts of the enzyme. These were identified as adenosine and an acid degradation product of NADH (Miksic & Brown, 1977), on the basis of comparison with A_{280}/A_{254} peak height

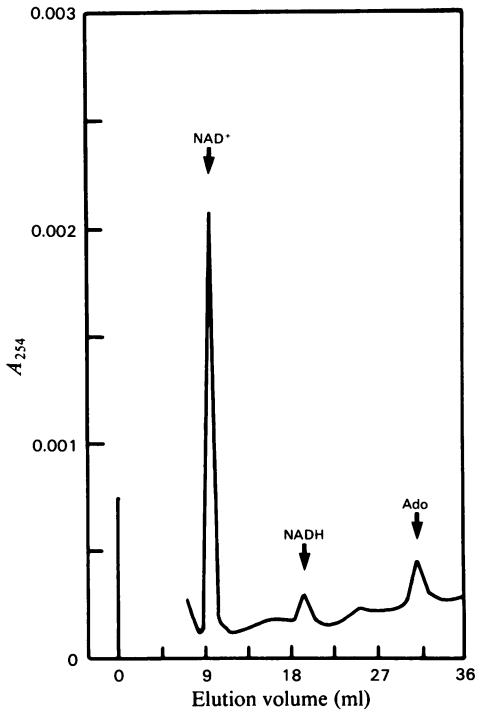

Fig. 3. *H.p.l.c. analysis of ligands bound to AdoHcyase*
Purified placental AdoHcyase was treated with 2M-HCl and analysed by h.p.l.c. as described in the Materials and methods section. The injected sample (equivalent to 5.7μg of protein) contained 94pmol of NAD^+, 20pmol of NADH and 30pmol of adenosine (Ado).

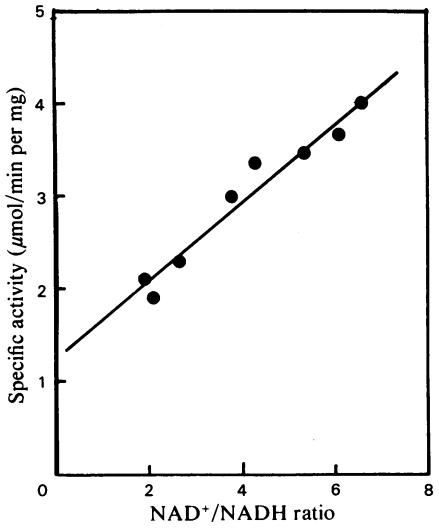

Fig. 4. *Relationship between the ratio of NAD^+/NADH and specific activity of AdoHcyase*
The activity was measured in the AdoHcy synthesis reaction, and results for eight separate preparations of AdoHcyase are shown.

ratios and elution volumes of adenosine and NADH standards treated in the same manner as enzyme. To confirm the identity of the NADH breakdown product, we prepared AdoHcyase containing radioactively labelled NAD^+ by growing CEM human lymphoblastoid cells in medium containing either [^{14}C]nicotinamide or [^{3}H]adenine [Materials and methods section and Hershfield *et al.*, 1982). Incubation with adenine arabinoside, which converts AdoHcyase-associated NAD^+ into NADH (Hershfield, 1980; Helland & Ueland, 1981; Chiang *et al.*, 1981; Hershfield *et al.*, 1982) shifted the position of both the nicotinamide-labelled and adenine-labelled NAD^+ peaks to the position of the NADH degradation product (results not shown). In these experiments we were also able to rule out the possibilities that inactivation by adenine arabinoside resulted in either covalent binding of a portion of the NAD^+ molecule to the enzyme (as might occur if the enzyme possessed an inherent auto-ADP-ribosyltransferase activity) or in release or cleavage of the NAD^+ (by an inherent NADase activity). Small amounts of adenine were found in two preparations of placental AdoHcyase (adenine is eluted between the peaks of NADH and adenosine in the h.p.l.c. system shown in Fig. 3). The adenine may have been derived from adenosine, since AdoHcyase from mouse liver has been shown to convert a variable fraction of bound adenosine into adenine (Ueland & Saebo, 1979).

Most preparations of placental AdoHcyase, obtained by both conventional and AdoHcy–agarose affinity chromatography, contained only 0.7–0.85mol of NAD^+/mol of subunit, and some as little as 0.5mol. The amounts of enzyme-associated NADH and adenosine were each in the range 0.1–0.35mol/mol of subunit. The mean$\pm$S.D. values (mol/mol of subunit) for eight separate preparations of affinity-purified placental AdoHcyase were: NAD^+, 0.76 ± 0.15; NADH, 0.21 ± 0.07; adenosine, 0.21 ± 0.05. The sum of NAD^++NADH was 0.97 ± 0.11mol/mol of subunit. The specific activity of these enzyme preparations, each of which was essentially homogeneous by sodium dodecyl sulphate/polyacryamide-gel electrophoresis, correlated with the enzyme-bound NAD^+/NADH ratio (Fig. 4). The presence of approximately equal amounts of NADH and adenosine may not be fortuitous, insofar as it has been postulated that reduction of enzyme-bound NAD^+, which accompanies adenosine binding, converts AdoHcyase into a 'closed' form that does not release bound molecules (Abeles *et al.*, 1982).

Binding of adenosine to AdoHcyase

Rat liver AdoHcyase has been shown to bind 1mol of adenosine/mol of subunit (Fujioka &

Takata, 1981; Gomi & Fujioka, 1982). However, the stoichiometry of adenosine binding has not been reported, or has been found to be variable and less than 1 mol/mol of subunit for the enzyme from other sources (Abeles *et al.*, 1982). We have previously noted variability among preparations of placental AdoHcyase in the capacity to bind radiolabelled adenosine, in the rate of adenosine binding and in the sensitivity to inactivation by adenosine (Hershfield *et al.*, 1979*b*). The maximum amount of radiolabelled adenosine that could be bound has ranged from 0.6 to 0.9 mol/mol of subunit. This variability might be accounted for by the presence of 0.1–0.4 mol of tightly bound unlabelled adenosine/mol of subunit (see above), or by an inability of catalytically inactive enzyme to bind adenosine. To examine these possibilities directly, AdoHcyase was preincubated at 37°C for 3 h with either no additions or with 90 μM unlabelled adenosine, 4′,5′-dehydroadenosine or adenine, followed by dialysis to remove loosely bound ligands. Compared with the untreated and adenine-treated samples, both the adenosine-treated and 4′,5′-dehydroadenosine-treated enzymes had lost about 95% of their catalytic activity (Fig. 5*b*). H.p.l.c. analysis showed that 90% of the cofactor present on the inactivated preparations was in the reduced form. The time course of [^{3}H]adenosine binding (Fig. 5*a*) was similar for all four preparations, with about half of the labelled adenosine becoming bound within 20 min, followed by a slower binding of an equivalent amount by 4 h, after which no further binding was observed. The adenosine-treated and 4′,5′-dehydroadenosine-treated samples bound only 37% as much [^{3}H]-adenosine as did the control and adenine-treated preparations. However, h.p.l.c. analysis revealed that the total amount of adenosine associated with the four enzyme preparations was identical, 0.9 mol/mol of subunit. These results show that catalytically inactive enzyme has the same total adenosine-binding capacity as active enzyme, and

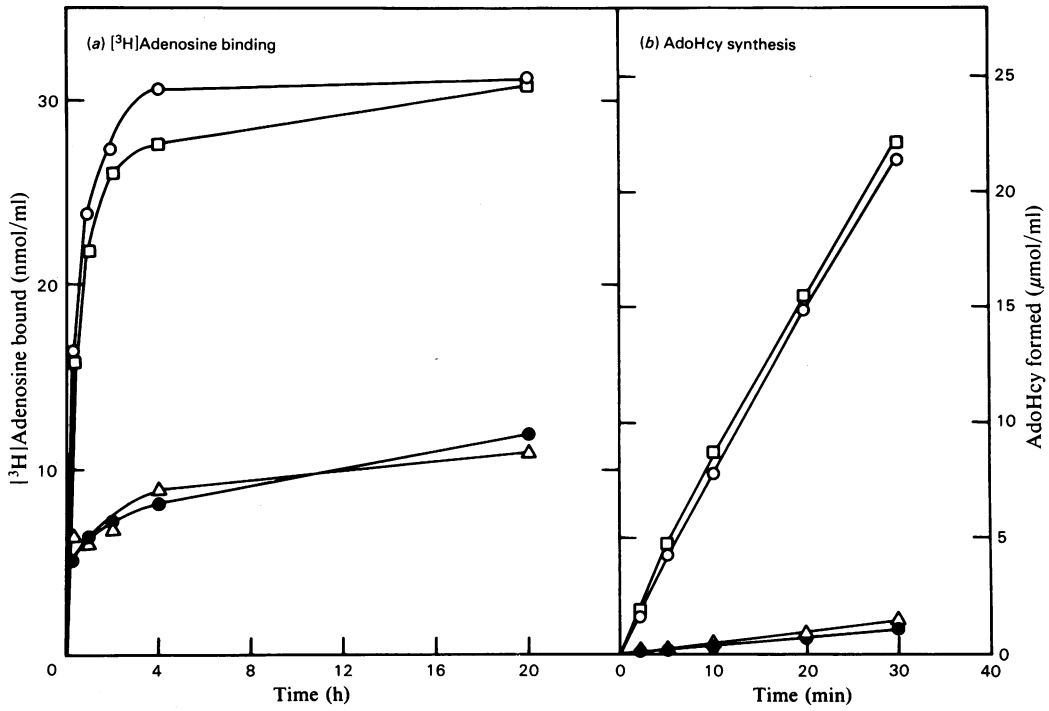

Fig. 5. *Effect of prior exposure to adenosine and 4′,5′-dehydroadenosine on AdoHcyase catalytic activity and adenosine-binding capacity*

Purified placental AdoHcyase (40 μg) was incubated for 3 h at 37°C in 0.11 ml (total volume) of 25 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-dithiothreitol and 1 mM-EDTA and no other additions (control), 90 μM-adenine, 90 μM-adenosine or 90 μM 4′,5′-dehydroadenosine, and then dialysed for 14 h at 4°C against 10 mM-potassium phosphate buffer, pH 7.0. Samples of the dialysed preparations were diluted 25-fold into 25 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-dithiothreitol and 1 mM-EDTA, and these were assayed for enzymic activity (AdoHcy synthesis) (*b*) and for content of bound ligands by h.p.l.c. (discussed in the text). The remainder was used to assay binding of [^{3}H]adenosine (20 μM, 1335 c.p.m./pmol) (*a*). ○, No additions (control); □, adenine-treated; △, 4′,5′-dehydroadenosine-treated; ●, adenosine-treated.

suggest that differences among preparations in capacity to bind labelled adenosine are due to differences in amounts of tightly bound unlabelled adenosine.

As noted in a preliminary report (Hershfield, 1978), dissociation of the AdoHcyase–[^{3}H]adenosine complex is biphasic. Biphasic binding or dissociation of adenosine has also been observed with AdoHcyase from mouse liver (Ueland & Doskeland, 1978) and yellow-lupin seed (Jakubowski & Guranowski, 1981). Two types of AdoHcyase–adenosine complexes could also be demonstrated in studies of adenosine binding at 0°C and 37°C. In 20min at 0°C the enzyme bound about 0.3mol of [^{3}H]adenosine/mol of subunit, increasing to a maximum of 0.4mol/mol in 7h, during which no enzymic activity was lost. A portion shifted to 37°C after 2h at 0°C bound 0.9mol of adenosine/mol of subunit, accompanied by loss of 97% of enzymic activity (results not shown).

We have reported that some preparations of placental AdoHcyase are quite insensitive to inactivation by adenosine (Hershfield *et al.*, 1979*b*). Adenosine inactivated bovine liver AdoHcyase (Chiang *et al.*, 1981) but had no effect on the rat liver enzyme (Fujioka & Takata, 1981). Studies with purified AdoHcyase from mouse liver (Ueland *et al.*, 1978) and rat liver (Gomi & Fujioka, 1982), and our own studies with the human placental enzyme (M. S. Hershfield, V. N. Aiyar, R. Premakumar & W. C. Small, unpublished work), indicate that binding of adenosine blocks the reaction of iodoacetamide and 5,5′-dithiobis-(2-nitrobenzoic acid) with cysteine residues that are essential for catalytic activity. Thus variability in sensitivity to inactivation by adenosine might be due to modification of cysteine residues during enzyme purification or storage. This possibility is suggested by the experiment shown in Fig. 6, in which we examined binding of [^{14}C]adenosine to AdoHcyase that had first been dialysed against buffers that contained or lacked dithiothreitol. In the presence of dithiothreitol binding reached a maximum in about 6h, associated with complete intactivation of the enzyme. Removal of dithothreitol had little effect on initial enzyme activity, but it greatly lowered the rate of adenosine binding. A fraction of the dithiothreitol-free preparation, representing 20–25% of the initial enzymic activity, was resistant to inactivation.

Abeles *et al.* (1982) reported that excess adenosine or 2′-deoxyadenosine caused the reduction of a maximum of two out of four enzyme-bound NAD^+ molecules. They suggested that two of the enzyme subunits were therefore not involved in catalysis and might serve a regulatory role. Doskeland & Ueland (1982) have reported that both mouse and

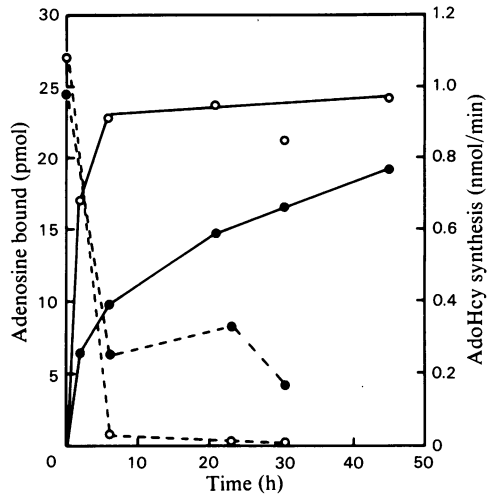

Fig. 6. *Effect of removal of dithiothreitol on binding of adenosine to AdoHcyase*

Two 84μg samples of purified AdoHcyase were dialysed for 17h at 4°C, one against 25mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA and 0.02% NaN_3 (to prevent bacterial growth during subsequent steps), and the second against the same buffer containing 1mM-dithiothreitol. After dialysis, deoxycoformycin and [^{14}C]-adenosine were added to give final concentrations of 2μM and 25μM respectively. The reaction mixtures (350μl) were then incubated at 37°C, and at the times indicated duplicate samples were removed from each for measurement of the amount of [^{14}C]adenosine that had become bound by Sephadex G-25 gel filtration (——) and enzymic activity (AdoHcy synthesis) (----). ○, Plus dithiothreitol; ●, no dithiothreitol.

bovine liver AdoHcyases give two bands of slightly differing mobility, present in a 1:1 ratio, on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and suggested that the enzymes are composed of two kinds of subunits arranged in an A_2B_2 manner. In contrast with the findings reported by Abeles *et al.* (1982), treatment of placental AdoHcyase with adenosine, 2′-deoxyadenosine or adenine arabinoside can cause reduction of 0.8–1mol of NAD^+/mol of subunit. Nor have we found any physical evidence of non-identical subunits. Evidence that rat liver AdoHcyase is composed of structurally identical and catalytically equivalent subunits has been reported by Gomi *et al.* (1985). The finding of a single genetic locus for AdoHcyase (Hershfield & Francke, 1982; Mohandas *et al.*, 1984) suggests that there may be a single structural gene that encodes a single peptide of which the native enzyme is composed. It is possible, however, that the enzyme arose through a process that involved

contiguous duplication of a coding region, followed by evolution into two linked genes encoding distinct peptides containing some conserved and some dissimilar regions. Evaluation of these possibilities must await information concerning the sequence of the enzyme and its structural gene(s).

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