Covalent labelling of ligand binding sites of human placental *S*-adenosylhomocysteine hydrolase with 8-azido derivatives of adenosine and cyclic AMP

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S-Adenosylhomocysteine hydrolase (AdoHcyase) has previously been identified as a cytoplasmic adenosine and cyclic AMP binding protein. In order to examine the relationship between the adenosine and cyclic AMP binding sites on this enzyme we have explored the use of 8-azido analogues of adenosine and cyclic AMP as photoaffinity reagents for covalently labelling AdoHcyase purified from human placenta. 8-Azidoadenosine (8-N₃-Ado), like adenosine, inactivated AdoHcyase, and the rate of inactivation was greatly increased by periodate oxidation. In addition, 8-N₃-Ado was found to participate in the first step in the catalytic mechanism for AdoHcyase, resulting in conversion of enzyme-bound NAD⁺ to NADH, although it was not a substrate for the full enzyme-catalysed reaction. Radioactively labelled 8-N₃-Ado, its periodate-oxidized derivative and 8-azidoadenosine 3', 5'-phosphate (8-N₃-cAMP) bound specifically to adenosine binding sites on AdoHcyase and, after irradiation, became covalently linked to the enzyme. Photoaffinity-labelled enzyme could be precipitated by monoclonal antibody to human AdoHcyase. Two observations suggested that cyclic AMP and adenosine bind to the same sites on AdoHcyase. First cyclic AMP and adenosine each blocked binding of both radioactively labelled 8-N₃-Ado and 8-N₃-cAMP, and second, digestion with V8 proteinase generated identical patterns of peptides from AdoHcyase that had been photolabelled with [³²P]8-N₃-cAMP and [³H]8-N₃-Ado. Binding sites for cyclic AMP on AdoHcyase were found to differ functionally and structurally from cyclic AMP binding sites on the R₁ regulatory subunit of cyclic AMP-dependent protein kinase.

INTRODUCTION

S-Adenosylhomocysteine hydrolase (EC 3.3.1.1; AdoHcyase) catalyses the reversible hydrolysis of AdoHcy, a product and competitive inhibitor of *S*-adenosylmethionine-dependent transmethylation reactions, to adenosine plus L-homocysteine (de la Haba & Cantoni, 1959). Palmer & Abeles (1976, 1979) found that the enzyme possesses tightly, but not covalently, bound NAD⁺ cofactor, which is essential for catalytic activity. Studies from this laboratory (Hershfield & Kredich, 1978) showed that AdoHcyase can bind adenosine with high affinity, and identified AdoHcyase as the so-called 'cyclic AMP/adenosine binding protein' or 'adenine analogue binding protein' that had been isolated from several tissues (Yuh & Tao, 1974; Sugden & Corbin, 1976; Ueland & Doskeland, 1977; Doskeland & Ueland, 1977; Olsson, 1978; Ueland, 1982). We have also shown that several analogues of adenosine, notably 2'-deoxyadenosine and adenine arabinoside, bind tightly to AdoHcyase and cause its irreversible inactivation (Hershfield, 1979).

The ability to form stable complexes with NAD⁺, adenosine and its analogues and cyclic AMP has raised questions regarding the possible relatedness of AdoHcyase to other proteins that bind these ligands, and regarding possible functions related to ligand binding. Gabriel

(Gabriel, 1978; Gabriel & Van Lenten, 1978) has noted similarities in the mechanisms of reactions catalysed by a group of enzymes, including AdoHcyase, that contain tightly bound NAD⁺, which may indicate an origin distinct from the majority of cellular enzymes that catalyse exogenous NAD⁺-dependent redox reactions. AdoHcyase and adenosine deaminase (EC 3.5.4.4; ADA) catalyse sequentially the production and degradation of adenosine. Evidence both *in vitro* and *in vivo* indicates that the activity of AdoHcyase is physiologically dependent upon ADA since (i) deamination of adenosine is necessary to pull the thermodynamically unfavourable hydrolase reaction in the physiological direction of AdoHcy breakdown (de la Haba & Cantoni, 1959; Kredich & Martin, 1977; Kredich & Hershfield, 1979), and (ii) 2'-deoxyadenosine is an inactivator of AdoHcyase (Hershfield, 1979; Hershfield *et al.*, 1979a, 1983, 1984; Mitchell *et al.*, 1980). We have recently mapped the gene for AdoHcyase to the long arm of chromosome 20 (Hershfield & Francke, 1982; Mohandas *et al.*, 1984), which is also the location of the gene for ADA (Tischfield *et al.*, 1974; Philip *et al.*, 1980). Taken together these observations have led us to suggest the possibility of an evolutionary relationship between ADA and AdoHcyase: either that linkage of the evolving gene for AdoHcyase to the ADA locus was an advantage during evolution of

Abbreviations used: AdoHcy, *S*-adenosylhomocystein; AdoHcyase, *S*-adenosylhomocysteine hydrolase; 8-N₃-Ado, 8-azidoadenosine; 8-N₃-cAMP, 8-azidoadenosine 3',5'-phosphate; ADA, adenosine deaminase; SDS, sodium dodecyl sulphate; Ado-ox, periodate-oxidized adenosine.

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AdoHcyase, or that the two genes arose from a common sequence that encoded a primitive nucleoside-binding domain (Hershfield & Francke, 1982).

The function of cyclic AMP binding to AdoHcyase is not known. There is no evidence that cyclic AMP regulates AdoHcy catabolism in intact cells, or that AdoHcyase mediates biological effects of cyclic AMP. Cyclic AMP did not effect the activity of AdoHcyase from beef liver (Chiang *et al.*, 1981), but preincubation with cyclic AMP has recently been reported to cause reversible inactivation of AdoHcyase from *Dictyostelium* and from rabbit erythrocytes (Hohman & Vernon, 1984; Hohman *et al.*, 1984). Several reports have suggested that cyclic AMP and adenosine bind to different sites on AdoHcyase, which may indicate the existence of separate binding domains and distinct functions for the two ligands (Yuh & Tao, 1974; Sugden & Corbin, 1976; Ueland & Doskeland, 1977; Doskeland & Ueland, 1977; Olsson, 1978; Chiang *et al.*, 1981). It is possible that the cyclic AMP binding region of AdoHcyase is related to cyclic AMP binding sites of the regulatory subunits of cyclic AMP-dependent protein kinase, which are the major cyclic AMP binding proteins in eukaryotic cells.

As a step towards physical characterization of the adenosine- and cyclic AMP-binding sites of AdoHcyase we have investigated the possibility of covalently labelling the enzyme with photoactive derivatives of these compounds, 8-N₃-Ado and 8-N₃-cAMP.

MATERIALS AND METHODS

Materials

Adenosine, homocysteine thiolactone, 8-N₃-cAMP, α -chymotrypsin and trypsin were obtained from Sigma. *Staphylococcus aureus* V8 proteinase was purchased from Miles Laboratories; GF/C glass fibre filters from Whatman Biochemicals, AdoHcy-agarose (SAH agarose) from BRL, and cyclic AMP-agarose from PL Biochemicals. [2-³H]8-N₃-Ado (16 Ci/mmol) and [2-³H]8-N₃-cAMP (19 Ci/mmol) were obtained from New England Nuclear, [8-¹⁴C]adenosine (50 mCi/mmol) from Moravsek Biochemicals and [³²P]8-N₃-cAMP (87 Ci/mmol) from ICN. 8-N₃-Ado was prepared from adenosine as described (Holmes & Robins, 1965). Periodate oxidation of 8-N₃-Ado (both radioactive and non-radioactive) was performed as described for ATP by Clertant & Cuzin (1982). The reaction mixture was diluted with water and used immediately after preparation.

Enzyme purification

AdoHcyase was assayed in the direction of AdoHcy synthesis (Hershfield & Kredich, 1978; Hershfield, 1979). Human placental AdoHcyase was purified to apparent homogeneity by AdoHcy-agarose affinity chromatography as described (Hershfield *et al.*, 1985). Cyclic AMP binding protein R₁ was partially purified from leukaemic cells obtained by leukapheresis of a patient with chronic lymphocytic leukaemia, using DEAE-cellulose chromatography followed by affinity chromatography on cyclic AMP-agarose as described by Dills *et al.* (1979).

Assessment of 8-N₃-Ado as a substrate for AdoHcyase

The ability of AdoHcyase to catalyse the synthesis of 8-N₃-AdoHcy from L-homocysteine and 8-N₃-Ado, and to catalyse the conversion of 8-N₃-Ado to 4',5'-dehydro-8-N₃-Ado were assessed as follows. Reaction mixtures

(0.1 ml) contained 10 mM-potassium phosphate, pH 7.0, 200 μ M-8-N₃-Ado, 6.8 μ g of purified placental AdoHcyase (which had been dialysed against 10 mM-potassium phosphate, pH 7.0, to remove dithiothreitol) and 200 μ M-L-homocysteine (L-homocysteine was omitted in assessing formation of 4',5'-dehydro-8-N₃-Ado). AdoHcyase was omitted from control reaction mixtures, and in parallel reactions 8-N₃-Ado was replaced by 200 μ M-adenosine. Incubation was carried out in the dark at 37 °C for 40 min and terminated by addition of HClO₄ to a final concentration of 0.4 M, followed by centrifugation at 4 °C for 2 min in a Fisher microcentrifuge. Aliquots of reaction mixtures were analysed by h.p.l.c. using a C₁₈ μ Bondapak reversed phase column (Waters) eluted with 15% (v/v) ethanol in water at a flow rate of 1.0 ml/min. Effluent was monitored at 280 and 254 nm using a Waters 440 UV detector. Elution volumes were as follows: AdoHcy, 4.0 ml; adenosine, 4.8 ml; 4',5'-dehydroadenosine, 6.6 ml; 8-N₃-Ado, 9.0 ml. The ratio of peak heights at 280 nm to peak height at 254 nm for adenosine and its derivatives was 0.19; the ratio for 8-N₃-Ado was 2.81. Under the conditions of analysis we estimate that at little as 20 pmol, or 0.5–1% of the substrate injected, of 8-N₃-AdoHcy or 4',5'-dehydro-8-N₃-Ado could have been detected, assuming that the elution volumes and spectral characteristics of these compounds would bear the same relationship to 8-N₃-Ado as the analogous compounds bear to adenosine.

Photoaffinity labelling

Proteins in phosphate buffer (50 mM, pH 6.8) were preincubated for 15 min at 37 °C in the presence or absence of an unlabelled nucleoside. Radioactive photoaffinity ligand was added and incubation continued in the dark for 15–60 min. Reaction mixtures were then placed on ice and irradiated for 5 or 10 min at a distance of 8 cm, using a UVG 11 Mineralight operating at 254 nm. Covalent incorporation of radioactivity was analyzed by a glass fibre filter assay using 10% (w/v) trichloroacetic acid precipitation at 0 °C, and by SDS/polyacrylamide-gel electrophoresis. Total binding was monitored in aliquots of unirradiated reaction mixtures by determining radioactivity that was eluted in the void volume after gel filtration chromatography on PD 10 (Sephadex G-25; Pharmacia) columns. Immunoprecipitation of affinity-labelled AdoHcyase with monoclonal antibody B2A3 to human placental AdoHcyase, using *Staphylococcus* to precipitate the immune complex, was carried out as described (Hershfield & Francke, 1982).

Peptide mapping of affinity-labelled proteins was carried out by limited proteolysis as described by Cleveland *et al.* (1977). Samples of labelled proteins, as well as washed staphylococcal immunoprecipitates, were heated at 100 °C for 2 min in SDS sample gel buffer and analysed by SDS/mercaptoethanol/polyacrylamide-gel electrophoresis as described by Laemmli (1970). Fluorography of ³H-labelled proteins in gels was performed as described by Bonner & Laskey (1974). Unlabelled protein bands on gels were stained with Coomassie Blue or AgNO₃ (Merril *et al.*, 1981).

RESULTS

Inactivation of AdoHcyase by 8-N₃-Ado and 8-N₃-Ado-ox

Ado-ox is a potent competitive inhibitor of AdoHcyase (Hoffman, 1979; Borchardt *et al.*, 1982). Borchardt

Fig. 1. Inactivation of AdoHcyase by 8-N₃-Ado and 8-N₃-Ado-ox

AdoHcyase (1.7 μg) was preincubated in 25 mM-potassium phosphate (pH 7.0)/1 mM-dithiothreitol/1 mM EDTA/1 μM-deoxycoformycin at 37 °C in the presence of HIO₄, 8-N₃-Ado, or 8-N₃-Ado-ox. At the indicated times aliquots were removed and diluted 10-fold into reaction mixtures for measuring AdoHcyase activity (Hershfield, 1979); 100% activity 1.32 nmol of AdoHcy formed/min. ○, 1.34 μM-HIO₄; ●, 24 μM-8-N₃-Ado; □, 0.048 μM-8-N₃-Ado-ox; ■, 0.060 μM-8-N₃-Ado-ox; △, 0.096 μM-8-N₃-Ado-ox; ▲, 0.162 μM-8-N₃-Ado-ox.

et al. (1982) have suggested that the dialdehyde of adenosine produced by periodate oxidation may form a Schiff base with an amino acid residue, possibly lysine, in the enzyme active site. These observations suggested that Ado-ox itself might be used to label AdoHcyase covalently. However, we were unable to detect cold acid- or SDS-stable labelling of placental AdoHcyase after treatment with [³H]Ado-ox, either alone or followed by NaBH₄. Therefore we attempted to develop more effective reagents for covalently modifying the enzyme by addition of an azido group to the 8 position of the purine ring of adenosine. The resulting compound, 8-N₃-Ado, like adenosine itself (Hershfield *et al.*, 1979b), caused slow inactivation of AdoHcyase, which was greatly enhanced (about 500-fold) by periodate oxidation (Fig. 1).

The finding that 8-N₃-Ado inactivates AdoHcyase suggested that it interacts with the active site of the enzyme. We explored this possibility further by asking whether 8-N₃-Ado could serve as a substrate for AdoHcyase, either in the complete reaction, condensation with L-homocysteine to form 8-N₃-AdoHcy, or in a partial reaction. According to the mechanism for AdoHcy synthesis proposed by Palmer & Abeles (1976, 1979) the first step in catalysis involves oxidation of the 3'-OH group of adenosine to form 3'-oxoadenosine with conversion of enzyme-bound NAD⁺ to NADH. This is followed by abstraction of a proton from the 4' carbon atom by a base on the enzyme, leading to elimination of water between the 4' and 5' carbon atoms, forming 4',5'-dehydro-3'-oxoadenosine. The latter can react with homocysteine to form AdoHcy, or can undergo reduction of the 3'-oxo group to form 4',5'-dehydroadenosine (with oxidation of NADH to NAD⁺). 4',5'-Dehydroadenosine accumulates when adenosine is incubated with placental AdoHcyase in the absence of homocysteine (Hershfield *et al.*, 1985). Thus, we assessed three reactions involving AdoHcyase and 8-N₃-Ado: (a) conversion of enzyme-bound NAD⁺ to NADH, measured as a decrease in

Table 1. Effects of ligands on NAD⁺ content of AdoHcyase

AdoHcyase (6.8 μg) was incubated in a reaction mixture (0.05 ml) containing 10 mM-potassium phosphate pH 7.0, and the indicated compounds (200 μM each) in the dark at 37 °C for 40 min. The reaction was terminated by addition of 0.01 ml of 2 M-HClO₄. After centrifugation at 4 °C, the NAD⁺ content of the reaction mixtures was analysed by h.p.l.c. (Hershfield *et al.*, 1985) on a C₁₈ column eluted with 50 mM-sodium acetate (pH 3.9)/5% (v/v) methanol at a flow rate of 1 ml/min. NAD⁺ was eluted in a volume of 6.4 ml.

Additions	NAD ⁺ (pmol)
None	125.5
8-N ₃ -Ado	64.1
Adenosine	9.4
8-N ₃ -cAMP	116.2
Cyclic AMP	123.1

NAD⁺ extractable from enzyme; (b) formation of 4',5'-dehydro-8-N₃-Ado in the absence of homocysteine; and (c) formation of 8-N₃-AdoHcy in the presence of homocysteine.

We observed no conversion of 8-N₃-Ado to either 8-N₃-AdoHcy or to 4',5'-dehydro-8-N₃-Ado, while under the same conditions conversion of adenosine to the analogous products was easily demonstrable (results not shown). However, 8-N₃-Ado did cause a 50% decrease in enzyme-associated NAD⁺ (Table 1). This decrease was accompanied by a corresponding increase in h.p.l.c. peaks derived from breakdown products of NADH, which is labile in acid, as we have demonstrated elsewhere in studies of AdoHcyase inactivation by adenine arabinoside (Hershfield *et al.*, 1982, 1985) (results not shown). Under the same conditions adenosine caused nearly complete reduction of cofactor, while neither cyclic AMP or 8-N₃-cAMP, which lack 3'-OH groups, appreciably affected enzyme NAD⁺ content.

Binding of 8-N₃-Ado and 8-N₃-cAMP

In initial experiments we found that in the presence of thiol reagents, which react with the 8-azido group, we were unable to achieve acid- or SDS-stable photolabelling of AdoHcyase. This made it necessary to conduct experiments in the absence of thiols. As we have shown (Hershfield *et al.*, 1985), in the absence of dithiothreitol binding of radiolabelled adenosine to purified placental AdoHcyase proceeds at < 10% of the rate observed in its presence, and results in incomplete occupancy of available adenosine-binding sites. Thus, total binding of labelled 8-azido derivatives of adenosine and cyclic AMP to AdoHcyase in the absence of irradiation was unavoidably inefficient, proceeding to no more than 0.15 molecules/subunit.

Noncovalent binding of [³H]8-N₃-Ado to AdoHcyase, measured by gel filtration of unirradiated reaction mixtures, was inhibited > 90% by preincubation of enzyme with either excess unlabelled adenosine or cyclic AMP (Table 2). Similarly, unlabelled Ado-ox effectively prevented binding of [³H]8-N₃-Ado-ox to AdoHcyase. Binding of [³²P]8-N₃-cAMP was inhibited 85–90% by both unlabelled adenosine and cyclic AMP. These results indicate that the 8-azido derivatives of both adenosine cyclic and AMP bind to specific adenosine and cyclic AMP sites on AdoHcyase.

Table 2. Binding of [³H]8-N₃-Ado and [³²P]8-N₃-cAMP by AdoHcyase

AdoHcyase, 208 µg/ml in 50 mM-potassium phosphate (pH 6.8)/1 mM-EDTA was incubated for 30 min at 37 °C with the indicated concentration of adenosine or cyclic AMP. Then [³H]8-N₃-Ado or [³²P]8-N₃-cAMP was added to final concentrations of 0.39 M or 0.33 µM, respectively. Incubation was continued for 60 min in the dark, after which aliquots containing 10.4 µg of AdoHcyase were removed and diluted to 0.5 ml in reaction buffer containing 0.1 mg of bovine serum albumin/ml, and then passed over PD-10 Sephadex G-25 (Pharmacia) gel filtration columns for measurement of radioactivity bound. Values for 100% binding in the absence of unlabelled adenosine or cyclic AMP were 39000 c.p.m. for [³H]8-N₃-Ado, and 63000 c.p.m. for [³²P]8-N₃-cAMP. Background was determined in reactions from which AdoHcyase was omitted.

Additions	Amount bound (% of control)
[³ H]8-N ₃ -Ado	100
[³ H]8-N ₃ -Ado + adenosine (40 µM)	10.0
[³ H]8-N ₃ -Ado + adenosine (108 µM)	3.0
[³ H]8-N ₃ -Ado + adenosine (200 µM)	<0.1
[³ H]8-N ₃ -Ado + cyclic AMP (50 µM)	10.0
[³ H]8-N ₃ -Ado + cyclic AMP (200 µM)	8.0
[³² P]8-N ₃ -cAMP	100
[³² P]8-N ₃ -cAMP + adenosine (170 µM)	10.0
[³² P]8-N ₃ -cAMP + cyclic AMP (50 µM)	3.6

In a number of experiments we found that 5–14% of the specifically bound radiolabelled 8-N₃-Ado, 8-N₃-Ado-ox or 8-N₃-cAMP remained associated with the enzyme in an acid-precipitable form following irradiation. This yield, which is typical for photoincorporation of azido compounds (Potter & Haley, 1983), could not be improved by varying the time of irradiation, or by the use of high-intensity irradiation at 320 nm for periods from 20 s to 5 min at 0 °C. Of the label incorporated covalently after irradiation, 40–80% was blocked by unlabelled adenosine or cyclic AMP. The reason(s) for variability among experiments is unclear. At comparable concentrations (100–200-fold in excess of the labelled azido analogue) uridine, which does not bind to AdoHcyase, had no effect on either noncovalent binding or photolabelling. A presently unexplained but consistent finding was that unlabelled 8-azidoadenosine 5-phosphate (but not 8-N₃-Ado or 8-N₃-cAMP), present at 10–100 µM, stimulated covalent incorporation of 0.5–2 µM-[³H]8-N₃-Ado by 2–3-fold; this photolabelling was not blocked by up to 100 µM unlabelled adenosine. In contrast to this stimulation by 8-azidoadenosine 5'-phosphate, comparable concentrations of AMP blocked both noncovalent binding and photolabelling by [³H]8-N₃-Ado by 50%.

We observed no covalent photolabelling by either [³H]8-N₃-Ado or [³H]8-N₃-Ado-ox of ovalbumin, or of highly purified preparations of ADA (human or calf) or R₁ cyclic AMP binding protein. However, both ligands were incorporated nonspecifically into placental proteins other than AdoHcyase. Fig. 2 is an SDS gel showing that proteins that were covalently labelled by [³H]8-N₃-Ado-ox in the pooled fractions from the DEAE-cellulose purification step, and in the nearly homogeneous

Fig. 2. Specificity of photolabelling of AdoHcyase with [³H]8-N₃-Ado-ox

Affinity-purified AdoHcyase (8 µg, lanes 1 and 2) or a crude placental extract (11.6 µg, lanes 3 and 4) was incubated for 15 min at room temperature in 50 mM-potassium phosphate (pH 7.0)/5 µM-deoxycoformycin (to prevent deamination of unlabelled adenosine) in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 100 µM-unlabelled adenosine. Then the samples were incubated for 15 min in the dark after addition of 5 µM-[³H]8-N₃-Ado-ox, after which they were placed on ice and irradiated for 5 min as described in the Materials and methods section. The samples were then analysed by SDS/mercaptoethanol/10%-polyacrylamide-gel electrophoresis followed by fluorography.

AdoHcyase obtained after elution of this material from AdoHcy-agarose with AdoHcy (Hershfield *et al.*, 1985). In the material applied to the affinity column the most prominently labelled bands had *M_r* values of 72000 and 11000. Preincubation with excess unlabelled Ado did not block labelling of these bands, but greatly decreased incorporation into the *M_r* 47000 AdoHcyase band in the affinity-purified material. The labelled *M_r* 47000 band was specifically precipitated with monoclonal antibody to placental AdoHcyase, whereas there was no reaction of the *M_r* 72000 or 11000 bands with antibody (results not shown).

Fig. 3. Photolabelling of AdoHcyase and R_1 cyclic AMP-binding protein with $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$

Placental AdoHcyase (17.5 μg) or R_1 cyclic AMP binding protein (4.8 μg , partially purified from chronic lymphocytic leukaemia cells) were incubated for 30 min at 37 °C in 50 μl reaction mixtures containing 50 mM-potassium phosphate, pH 6.9, in the presence or absence of 100 μM unlabelled adenosine cyclic or AMP, as indicated. Incubation was continued for another 30 min after addition of $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$, 1.5 μM . The samples were then irradiated at 0 °C for 10 min at a distance of 8 cm and analysed by SDS/mercaptoethanol/10%-polyacrylamide-gel electrophoresis followed by autoradiography. Lanes 1–3, AdoHcyase; lanes 4–6, R_1 . Multiple labelled bands in the R_1 preparation represent proteolytic products of R_1 (Sharma, 1982; Flockhardt & Corbin, 1982).

Binding sites for 8- $\text{N}_3\text{-Ado}$ and 8- $\text{N}_3\text{-cAMP}$ on AdoHcyase and the R_1 subunit of cyclic AMP-dependent protein kinase

As discussed above, both adenosine cyclic and AMP partially, and in some experiments nearly completely, blocked photolabelling of purified placental AdoHcyase by ^3H and ^{32}P -labelled 8- $\text{N}_3\text{-cAMP}$ (shown for $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$ in Fig. 3, lanes 1–3). In contrast, cyclic AMP, but not adenosine, blocked covalent incorporation of labelled 8- $\text{N}_3\text{-cAMP}$ into R_1 and into proteolytic fragments derived from R_1 (Fig. 3, lanes 4–6), while adenosine was without effect, consistent with the finding that R_1 was not photolabelled by $[^3\text{H}]8\text{-N}_3\text{-Ado}$. As with $[^3\text{H}]8\text{-N}_3\text{-Ado}$ -labelled AdoHcyase, the enzyme labelled with $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$ could be precipitated with monoclonal antibody to AdoHcyase (Fig. 4). Antibody to AdoHcyase did not react with photoaffinity-labelled R_1 (results not shown).

Fig. 4. Immunoprecipitation of $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$ -labelled AdoHcyase

Purified placental AdoHcyase (80 μg) was photolabelled at room temperature in 0.1 ml of 50 mM-potassium phosphate, pH 6.9, containing 1.5 μM - $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$, followed by irradiation as described in Fig. 3. After addition of 0.1 ml of 10 mM-potassium phosphate (pH 7.0)/0.15 M-NaCl (P_i/NaCl) containing 0.1% bovine serum albumin, aliquots containing 10 μg of AdoHcyase were incubated overnight at 4 °C in the absence or presence of B2A3 monoclonal antibody to placental AdoHcyase (Hershfield & Francke, 1982) (3.6 or 36 μg). Immune complexes were precipitated with 40 μl of a 10% suspension of protein A-Sepharose (Sigma). Pellets were washed with P_i/NaCl containing 0.05% Nonidet P-40 (Sigma) ($3 \times 1\text{ ml}$), and then extracted with SDS/mercaptoethanol electrophoresis sample buffer (Cleveland *et al.*, 1977) for analysis on a 10%-polyacrylamide gel and autoradiography. Lane 1, $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$ -labelled AdoHcyase used for immunoprecipitation (5 μg); lanes 2, 4, extracts of protein A-Sepharose pellets of incubations containing 0 (control), 3.6 and 36 μg of B2A3 monoclonal antibody, respectively.

Fig. 5 V8 proteinase digests of photoaffinity-labelled R_1 cyclic AMP binding protein and AdoHcyase

(a) R_1 (14 μ g) was incubated for 60 min at room temperature with 0.38 μ M- $[^3\text{H}]8\text{-N}_3\text{-cAMP}$ in 50 mM-potassium phosphate, followed by photolysis on ice for 10 min. An aliquot of the labelled protein was mixed with V8 proteinase at a substrate: proteinase ratio of 10:1, and incubation was continued for 30 min at 37 $^\circ\text{C}$. The reaction mixture was then analysed by SDS/mercaptoethanol/15%-polyacrylamide gel electrophoresis, followed by fluorography. (b) AdoHcyase (17.5 μ g) was photolabelled with $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$ as described in the legend to Fig. 3. Then 16 μ g of the labelled protein was digested with 1 μ g of V8 proteinase at 37 $^\circ\text{C}$ for 30 min, analysed on a 15%-polyacrylamide gel containing SDS, and transferred to nitrocellulose (Towbin *et al.*, 1979), which was analysed by autoradiography. (a) Lane 1, undigested R_1 ; lane 2, V8-digested R_1 . (b) Lane 1, V8-digested AdoHcyase; lane 2, undigested AdoHcyase.

In a series of experiments we compared the radioactive peptides generated by digestion of denatured AdoHcyase and R_1 , each labelled with $[^3\text{H}]$ - or $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$, with trypsin, chymotrypsin and V8 proteinase. No obvious similarities in labelled peptide patterns were found, as shown for V8 digests of the two proteins in Fig. 5. It has been shown by several laboratories that native R_1 can readily be cleaved by proteinases to yield distinct fragments that possess the cyclic AMP binding site of the protein, evidence that R_1 is made up distinct domains (Sharma, 1982; Flockhardt & Corbin, 1981). In contrast, native AdoHcyase was highly resistant to cleavage by trypsin, chymotrypsin and V8 proteinase (results not shown).

The findings that adenosine and cyclic AMP were both effective in blocking the noncovalent binding and photoincorporation of labelled 8- N_3 -Ado and 8- N_3 -cAMP (Table 2 and Fig. 3, lanes 1–3) suggest that these compounds interact at the same site or sites on the

enzyme. If this is the case one would expect to generate the same pattern of peptides upon proteolysis of AdoHcyase that had been labelled with either 8- N_3 -Ado or 8- N_3 -cAMP. As shown in Fig. 6 we did find virtually identical patterns of radioactive peptides after V8 proteinase digestion of $[^{32}\text{P}]8\text{-N}_3\text{-cAMP}$ - and $[^3\text{H}]8\text{-N}_3\text{-Ado}$ -labelled AdoHcyase.

DISCUSSION

Although AdoHcyase is irreversibly inactivated by 2'-deoxyadenosine and adenine arabinoside with kinetics that suggest a suicide process (Hershfield, 1979) neither of these nucleosides binds covalently to the enzyme (Hershfield, 1979; Abeles *et al.*, 1982; Helland & Ueland, 1981). The binding of adenosine and cyclic AMP are also noncovalent. We therefore investigated the use of 8-azido derivatives of adenosine and cyclic AMP as photoaffinity reagents for labelling ligand binding sites of AdoHcyase. 8- N_3 -cAMP has been used extensively as a photoaffinity probe for the regulatory (R) subunits of cyclic AMP-dependent protein kinase (Sharma, 1982), and 8- N_3 -Ado has been used as a photoaffinity probe for a plasma membrane-associated glycoprotein thought to be a component of the nucleoside transport system of adipocytes (Rosenblit & Levy, 1980). In the absence of irradiation, binding of both these azido compounds to AdoHcyase was effectively blocked by unlabelled adenosine and unlabelled cyclic AMP, indicating specific interaction of each azido compound with adenosine and cyclic AMP binding sites on this enzyme.

Both 8- N_3 -Ado and its periodate-oxidized derivative, 8- N_3 -Ado-ox, caused inactivation of AdoHcyase, which suggests that they interact with the enzyme active site. This interpretation is supported by the finding that 8- N_3 -Ado caused the conversion of enzyme-bound NAD^+ to NADH, presumably accompanied by conversion of 8- N_3 -Ado to its 3'-oxo derivative. The catalytic reaction with 8- N_3 -Ado apparently cannot proceed further, since we observed no formation of 4',5'-dehydro-8- N_3 -Ado, or of 8- N_3 -AdoHcy upon incubation of AdoHcyase with 8- N_3 -Ado and homocysteine. These results suggest that the enzyme cannot remove the 4' proton from 3'-oxo analogue intermediate, the next step in the catalytic mechanism (Palmer & Abeles, 1979). A similar block in catalysis with adenosine has been shown to occur after modification of cysteine residues of rat liver AdoHcyase with iodacetamide (Gomi & Fujioka, 1982). Thus, it seems likely that an essential cysteine residue at the active site may be unable to interact with the 3'-oxo derivative of 8- N_3 -Ado.

Several studies of 'adenine analogue binding proteins' and 'cyclic AMP/adenosine-binding proteins', later identified as AdoHcyase, have suggested that the protein possesses distinct binding sites for adenosine and cyclic AMP, with adenosine able to bind to the cyclic AMP site, but not the converse (Yuh & Tao, 1974; Sugden & Corbin, 1976; Ueland & Doskeland, 1977, 1978; Doskel- & Ueland, 1977; Olsson, 1978). Thus, it was found that adenosine completely blocked cyclic AMP binding, but cyclic AMP only partially prevented adenosine binding. In addition, dissociation curves for adenosine showed slow and fast components, while dissociation of bound cyclic AMP gave rapid, first-order kinetics (Ueland & Doskeland, 1978). Similarly, we have observed fast and slow dissociating complexes between

Fig. 6. Comparison of the 8- N_3 -Ado and 8- N_3 -cAMP binding sites of AdoHcyase by proteolytic mapping

Aliquots (17.5 μ g) of AdoHcyase were incubated at 37 °C for 30 min in 50 mM-potassium phosphate containing either 5 μ M-[3 H]8- N_3 -Ado (a, c) or 1.5 μ M-[32 P]8- N_3 -cAMP (b, d), followed by photolysis on ice for 10 min. The samples were then mixed with an equal volume of 0.125 M-Tris/HCl, pH 6.8, containing 0.5% SDS, 10% (v/v) glycerol and 0.001% Bromophenol Blue and heated at 100 °C for 2 min. Aliquots containing 16 μ g of the labelled proteins were then digested with 1 μ g of V8 proteinase for 30 min at 37 °C, after which mercaptoethanol and SDS were added to give final concentrations of 10% (v/v) and 2% (w/v), respectively. After boiling for 2 min aliquots of the undigested (a, b) or digested (c, d) enzyme were run on SDS/15%-polyacrylamide slab gels. Protein bands were then transferred electrophoretically to a nitrocellulose sheet and stained as described (Towbin *et al.*, 1979). Lanes containing the labelled proteins were cut into 2 mm slices for scintillation counting. Results are presented as a percentage of the total radioactivity present in the lane; 100% values for 32 P and 3 H were 2380 and 1100 d.p.m., respectively.

adenosine and AdoHcyase from human lymphoblastoid cells and placenta (Hershfield *et al.*, 1979b, 1985). Although these data are consistent with the presence of two binding sites for adenosine, one of which binds cyclic AMP, we feel that another interpretation is possible. Abeles *et al.* (1982) have suggested that formation of NADH by AdoHcyase ligands with 3'-OH groups converts the enzyme to a 'closed form' that traps these compounds. Studies with cyclic AMP and adenine, each of which lacks a 3'-OH group, are consistent with this proposal: neither cyclic AMP (Table 1) nor adenine (Hershfield *et al.*, 1985) reduce NAD^+ when they interact with AdoHcyase, and both ligands dissociate rapidly from the enzyme (Ueland & Doskeland, 1978; Hershfield *et al.*, 1979b). Cyclic AMP may bind to the same sites on AdoHcyase as adenosine, but lacking a 3'-OH group it cannot form a tight complex, and it cannot prevent formation of a tight complex by adenosine; thus cyclic AMP will appear to block adenosine binding only partially.

Two observations of the present study suggest that cyclic AMP and adenosine do bind to the same sites on placental AdoHcyase. First, cyclic AMP and adenosine each blocked binding of both radioactive 8- N_3 -Ado and

8- N_3 -cAMP to placental AdoHcyase (Table 2). Second, digestion with V8 proteinase generated identical patterns of radioactive peptides from placental AdoHcyase that had been photolabelled with [3 H]8- N_3 -Ado and [32 P]8- N_3 -cAMP (Fig. 6). These results must be interpreted with caution because of the low efficiency of covalent labelling, and because covalent labelling was not completely specific. Nevertheless, in view of these findings, and in the absence of any other physical evidence regarding the binding sites of adenosine or cyclic AMP, we believe that the question of distinct binding sites for cyclic AMP and adenosine on AdoHcyase needs to be reconsidered.

Our results indicate that the binding sites for 8- N_3 -cAMP on AdoHcyase and the R_1 subunit of cyclic AMP-dependent protein kinase are functionally, as well as structurally, dissimilar. AdoHcyase could be photoaffinity labelled by both 8- N_3 -Ado and 8- N_3 -cAMP, whereas only the latter labelled R_1 . Both adenosine and cyclic AMP blocked the labelling of AdoHcyase by [32 P]8- N_3 -cAMP, while only cyclic AMP blocked labelling of R_1 by this compound. We also observed no similarity between proteolytic fragments derived from 8- N_3 -cAMP-labelled denatured AdoHcyase and R_1 . These results do not exclude regions of limited homology, but

taken together they suggest that extensive sequence conservation in these proteins is highly unlikely.

Radiolabelled 8-N₃-cAMP has been considered to be a specific probe for the regulatory subunits of cyclic AMP-dependent protein kinase. Since this compound also labels AdoHcyase, which has a subunit *M_r* very similar to that of R₁, labelling of R₁ and AdoHcyase may be difficult to distinguish. For example, Mandler *et al.* (1982) examined the effect of adenosine on the occupancy by cyclic AMP of binding sites on R₁ in intact human peripheral blood T lymphocytes. Cells were exposed to exogenous adenosine, after which they were harvested and lysed. Occupancy of R₁ with endogenous cyclic AMP was assessed by measuring the binding of [³²P]N₃-cAMP to an *M_r* 48 000 protein isolated by SDS/polyacrylamide-gel electrophoresis. An adenosine-dependent decrease in labelling of this protein was interpreted as showing that adenosine increased R₁ occupancy by endogenous cyclic AMP. However, these investigators may instead have observed adenosine-dependent inhibition of AdoHcyase labelling by 8-N₃-cAMP (e.g. Fig. 3). This possibility is not trivial, since exogenous adenosine has been shown to bind rapidly and stably to intracellular AdoHcyase (Ueland & Helland, 1983). In view of our present findings, some previous studies of R₁ labelling with radioactive 8-N₃-cAMP may have to be evaluated with caution. The use of specific antibodies to AdoHcyase or R₁ in such studies may be helpful in assuring proper identification of labelled proteins.

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