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AFFINITY CHROMATOGRAPHY OF AN S-ADENOSYLMETHIONINE-DEPENDENT METHYLTRANSFERASE USING IMMOBILIZED S-ADENOSYLHOMOCYSTEINE

PURIFICATION OF THE INDOLETHYLAMINE N-METHYLTRANSFERASES OF *PHALARIS TUBEROSA*

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SUMMARY

In the cases that have been studied so far, S-adenosylhomocysteine (SAH) is a powerful inhibitor of S-adenosylmethionine (SAM) binding to SAM-dependent methyltransferases. We deduced, from the available data on the binding of SAM and SAH analogues to SAM dependent methyltransferases, that linkage of SAH through the carboxyl group to an immobilized support would lead to a more general affinity adsorbent for SAM-dependent methyltransferases than linkage through other functional groups. This paper describes the synthesis of this affinity adsorbent and its use to purify the two indolethylamine N-methyltransferases of *Phalaris tuberosa*.

INTRODUCTION

Phalaris tuberosa, a pasture grass of southeast Australia, contains two indolethylamine N-methyltransferases. The primary indolethylamine N-methyltransferase (PIM) catalyzes the N-methylation of the primary amines

$$\text{Indolethylamine} + \text{SAM} \rightarrow \text{N-methylindolethylamine} + \text{SAH}$$

where the indolethylamine is tryptamine (T), or its 5-hydroxy (5-OHT) (serotonin), or its 5-methoxy (5-MeOT) derivative. The corresponding products are N-methyltryptamine (MT), or the 5-hydroxy (5-OHMT) or the 5-methoxy (5-MeOMT) derivative.

The secondary indolethylamine N-methyltransferase (SIM) catalyzes the N-methylation of the above N-methyltryptamines to the corresponding N,N-dimethyltryptamines [DMT, 5-OHDMT (bufotenin) and 5-MeODMT]. DMT and 5-Me-

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ODMT are the physiological products of this pathway in *P. tuberosa*, from tryptophan, although the ratio of DMT to 5-MeODMT varies with the particular strain; 5-OHDMT is present, in lower concentrations[1,2].

The enzymes were not separated by preparative chromatographic techniques (gel filtration, CM- and DEAE-Sephadex ion-exchange, or hydroxyapatite) and the presence of two enzymes, in the 80-fold purified preparation, was deduced from the behavior of the enzyme activities towards various environmental effects (sodium chloride, urea, temperature, inactivation with time, pH optima and activity in solutions of various buffers, cations and anions). The PIM activities always correlated together and the SIM activities always correlated together[1,2].

Initial velocity and product inhibition studies showed that the mechanism for SIM was rapid equilibrium random bi-bi with formation of one dead-end complex[1,3] (not ordered as in ref. 2). The mechanism for PIM is likely to be the same due to the many other similarities of the enzymes[1–3], thus the enzymes will bind to the reactants independently and will bind to a suitable immobilized form of any of these four compounds.

Preliminary details of the affinity adsorbent described here have been reported before[3]. This paper is a full description of the affinity purification. Further details[1,2] will be described in subsequent papers.

MATERIALS AND METHODS

Buffers

All buffers contained ethylenediaminetetracetic acid (EDTA) (1 m*M*), mercaptoethanol (10 m*M*) and are 50 m*M* with respect to the buffering ion. The washing and eluting buffers for affinity chromatography contained bovine serum albumin (BSA) (250 μg/ml) and sodium chloride (150 m*M*). The fraction collecting tubes contained 0.2 vol. of 1 *M* Tris–HCl, pH 8.50 to restore the pH of the eluate to the original pH.

Enzyme preparation

This is described in ref. 1 and will be the subject of a future paper. Briefly, the shoots of ten-days old seedlings of *P. tuberosa* were homogenized, centrifuged (1 h at 140,000 *g*), the supernatant fractionated by ammonium sulphate, and the 50–60% saturation fraction chromatographed on DEAE-Sephadex. This gave an 80-fold purified preparation with 40% recovery of PIM and 30% recovery of SIM.

Enzyme assay

This is a modification of a previously described method[4]. The assay mixture containing: 80 μl enzyme in assay buffer (50 m*M* Tris–HCl, pH 8.50), 10 μl indolethylamine (100 nmole, final concentration 1 m*M*) and 10 μl [methyl-^{14}C] SAM (20 nmole, 10 pCi, final concentration = 200 μM) (total volume 100 μl) was incubated at 25° for 1 h in a capped scintillation vial. The reaction was terminated by addition of 1 *M* boric acid–sodium carbonate, pH 10.0 (200 μl) and then at convenience water (2 ml) was added (this lowers the blank level for the assay). Only the desired enzyme products were produced by the 80-fold purified preparation and these were extracted directly into scintillant [toluene, 10 ml containing 2,5-diphenyloxazole (4 g/l) and

1,4-di(2-(5-phenyloxazolyl))benzene (100 mg/l)], by shaking, after addition of the scintillant to the reaction mixture. The mixture could then be counted directly (boiled enzyme blank, 100 ± 30 pmole/h) or after decanting the scintillant following freezing of the mixture (boiled enzyme blank, 4 ± 2 pmole/h).

The presence of both enzymes in the enzyme preparation means that the assay for PIM will be systematically high because of the further methylation of the PIM reaction product by SIM. This led to a 30% overestimation of PIM activity and precluded kinetic analysis of this enzyme. However, since the ratio of PIM/SIM did not vary much in different enzyme preparations, this did not lead to any complication in estimating the presence of PIM activity.

Materials

Materials were purchased as follows: [methyl-^{14}C]-SAM, 0.5 mCi/mmole, CFA 428 from The Radiochemical Centre (Amersham, Great Britain); Agarose A-0.5 m from Bio-Rad Labs. (Richmond, Calif., U.S.A.); Sepharose 4B from Pharmacia (Uppsala, Sweden); tryptamine from BDH (Poole, Great Britain); other tryptamines were synthesized[5]; SAH, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide from Sigma (St. Louis, Mo., U.S.A.); cyanogen bromide, ethylenediamine, diaminodipropylamine from Mallinkrodt (St. Louis, Mo., U.S.A.) and *Phalaris tuberosa* seeds (commercial cultivar) from Rumseys Seeds (Sydney, Australia).

The activation and coupling were done by standard methods[6]. After cyanogen bromide activation, SAH was coupled directly to the gel through the N^6-group, by keeping the pH low (pH 7), this condition preferring coupling through the less basic N^6-group rather than the homocysteine amino group. Otherwise ethylenediamine or diaminodipropylamine were coupled to the activated gel, followed by carbodiimide coupling of the SAH. Gels (10 ml) were used, with side chain concentrations of 5–10 m*M* (as judged by titration), and SAH concentrations in the coupling mixture of 10–20 mg/10 ml column.

The proposed structure of the affinity column is shown in Fig. 1.

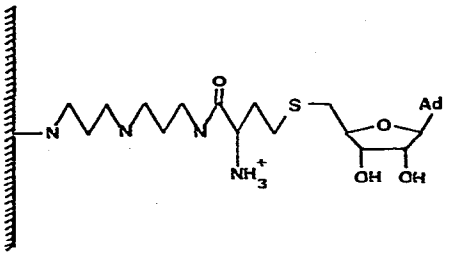

Fig. 1. The structure of the SAH affinity adsorbent.

RESULTS

Design of the affinity adsorbent

Earlier work has shown that SAH is a general inhibitor of SAM dependent methyltransferases, causing non-linearity in the time course of reaction[7], inhibiting tRNA[8,9] phenylethylamine, catechol and acetylserotonin[10] imidazole, acetylserotonin and homocysteine[11] catechol[12] and fatty acid carboxyl[13] methyltransferases.

Kinetic parameters and competition types have been determined for many enzymes and in all cases SAH was a strong inhibitor: tyramine and N-methyltyramine[14] phenylethylamine[10] catechol[15,16] histamine[17], N^2-guanine[18], protein II[19] arginine[20], lysine[21] indolethylamine (rabbit)[22], coniine[21], isoflavone[24] protein I[25], virus mRNA[26] and caffeic acid[27,28] methyltransferases.

SAM and SAH are co-factors for the bacterial DNA restriction modification enzymes and are allosteric effectors for some of the restriction endonucleases. Enzymically these methyltransferases differ from the other enzymes described here, through their low turnover number (in the order of minutes per reaction) and low K_m's[29–36].

SAH binds strongly to SIM[1,3] (competitive with respect to SAM binding, $K_I = 4\ \mu M$). PIM and SIM are not inhibited by adenosine (1 mM) or homocysteine (1 mM). We decided to try an affinity adsorbent containing an immobilized form of SAH. This adsorbent we hoped would also be of general use for purifying other SAM dependent methyltransferases. SAM was not tried as an affinity ligand because of its greater chemical and enzymic lability.

We then had to choose a suitable way of immobilizing SAH. There are three groups on SAH through which suitable linkages can be formed to an agarose gel. They are the 6-amino and the homocysteine amino and carboxyl groups. The nature of the SAM–SAH binding site has been investigated for three methyltransferases[11] using SAM analogues with one functional group missing. The homocysteine amino was required for binding to all methyltransferases, the acid group for only one enzyme and the purine 6-amino was not required for binding to any enzyme. Work on another enzyme showed that changing the base of SAH to guanine or cytosine produced a compound that was a poor inhibitor of the enzyme[37]. S-adenosylhomocysteamine (acid group removed) was still a good inhibitor.

The analogue with the ribose ring O replaced by $-CH_2-$ was inactive with catechol O-methyltransferase[16]. The 2-fluoro derivative was a poorer inhibitor of the enzyme than SAH ($K_I = 900\ \mu M$, as against 50 μM). The 6-hydroxy derivative was even a poorer ($K_I = 10$ mM) inhibitor.

An extensive study of SAH derivatives with five different methyltransferases[38] with modifications in the amino acid residue, the bases and the ribose residue, showed that the only derivatives which bound were the 3-deaza (tubercidinyl derivative), N^6-methyl-3-deaza, 3′-deoxy and arabinose analogues.

With N^2-guanine (in tRNA) methyltransferase[18] there was no inhibition when the 6-amino group of SAH was replaced by hydrogen or hydroxy. Monoalkylation, to 6-methylamino produced little change in inhibition, but dialkylation, to the 6-dimethylamino, destroyed inhibition[18,39]. The amino acid functions were not too important for this enzyme, D-SAH binding almost was well as L-SAH and S-adenosylthioenthanol, -propanediol and -propanoic acid still binding the enzyme. The amino group was more important than the acid group for binding as shown by the relative strength of binding of S-adenosylcysteamine, -ethanolamine and -propionic acids.

S-Tubercidinylhomocysteine (3-deaza-SAH) was a good inhibitor of catechol O-methyltransferase[40]. Changing the 5′-O to an amine or amide produced poor inhibitors[41].

Thus the acid function is the least stringently required functional group, of the three, for binding of SAH to most enzymes. Some enzymes seem to require the

correct hydrogen bonding at the N^6-site and others do not. The variability for the homocysteine amino group binding is even greater still. Thus for an affinity adsorbent, for general application, the preferred linkage of SAH to a gel would be through the acid group.

Non-enzymic hydrolysis of the glycosidic bond in SAH would be expected with time. The immobilized SAH would also be susceptible to nuclease or glycosidase activity in enzyme preparations. In fact, the binding of the enzymes to the gel decreased with time (the gels were unusable after about a month), but the reasons for this was not investigated.

Behaviour of the enzymes on the affinity adsorbents

Three gels were made, the first with SAH linked directly to the gel via the 6-amino group, the second with the acid function linked via an ethylenediamine side arm and the third with the acid function linked via a diaminodipropylamine side arm. The same concentration of SAH was used in these different gels.

All column buffers had high (150 m*M*) concentrations of sodium chloride to minimize ion-exchange effects. No differences were found in the chromatographic behaviour of the columns if A-0.5 m Agarose or Sepharose 4B were used as gel materials.

The enzymes were not retained by the first two gels under any conditions. The enzymes were retained by the third gel when applied in the optimal (assay) buffer (pH 8.5). Activity was eluted by decreasing the pH of the eluting buffers (pH 6.0, sodium maleate), but not by increasing the pH (pH 10.5, sodium carbonate). Under these conditions, irreproducible and very low recoveries of PIM (virtually undetectable) and SIM occurred, followed by a rapid loss of activity (within hours) after chromatography.

We assumed that in the more highly purified state, the enzymes were dena-

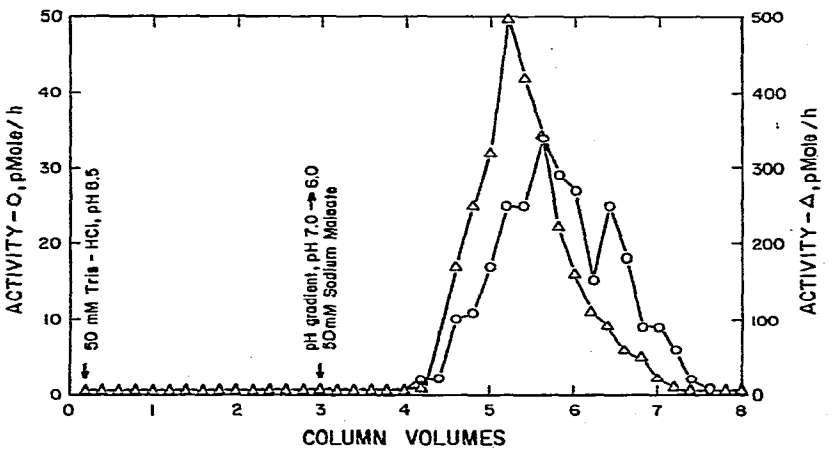

Fig. 2. Elution profile for the chromatography of the methyltransferases (200 μg total protein). ○, PIM, recovery 60–70%, substrate-tryptamine; △, SIM, recovery 125–150%, substrate-N-methyltryptamine; column, 1.2 × 10 cm; elution rate, 25 ml/h; eluting buffers, after washing with the initial buffer (pH 8.5, Tris–HCl) the enzymes were eluted with a linear gradient of sodium maleate (pH 7.0), start buffer, and sodium maleate (pH 6.0), finish buffer.

turing more quickly. Good recovery of the enzymes occurred when BSA was added to washing and eluting buffers and the eluates were returned to the assay pH and buffer. Under these conditions, 30% of PIM and 80% of PIM activity recovered from the columns, was present three days after elution. This is still less stable than the enzyme preparation before chromatography. No enzyme activity was eluted with up to 15 column volumes of initial buffer under these conditions. For a typical elution profile see Fig. 2.

DISCUSSION

The enzymes only bind to the gel with the long side arm (*i.e.*, when the ligand is "free"[6]) and not to the otherwise identical gel with the short side arm. This is consistent with the mechanism of retention being an affinity effect.

Due to the presence of protein in the eluting buffers, no estimate of the purification of the procedure is possible. However, the affinity adsorption of the enzymes followed by the sudden increase in lability of the enzymes on elution would indicate that a purification had been effected. The increase in activity of SIM following elution (150% recovery) also indicates that an inhibitor is being removed. The two enzymes were not separated by this procedure. This would indicate that the kinetic constants for the binding of PIM and SIM to SAH are similar. The elution profiles however are sufficiently different to show that they are two different enzymes (without a pH gradient the elution profiles were identical).

The enzymes were eluted under mild conditions (pH change). The enzymes require a high pH for optimal activity and are eluted from the affinity column by decreasing the pH but not by increasing the pH. This indicates the possibility that an uncharged amine group is required at the SAM–SAH-enzyme site. A possible contender for this group would be the homocysteine amine, particularly since this amine is required for bonding to many methyltransferases. If this is so, a decrease of pH of the eluting buffer by x pH units will lead to a decrease in the binding affinity of the SAH-enzyme complex by a factor of 10^x, thus leading to elution of methyltransferases with K_i's for SAH greater than 10^{-8} M, from affinity gels containing immobilized SAH at a concentration of 10^{-3} M, with a pH change of less than 5 units. If however, the pH optimum for a methyltransferase was low (around pH 5–7), the enzyme would probably require the protonated form of the amine (or a carboxy group) for binding, and the enzyme would be eluted by an increase in pH. Due to the ionic nature of SAH and hence its interactions with an enzyme, it seems likely that most enzymes would be eluted from an SAH gel by only a change in pH.

After we had communicated to Professor S. A. Brown, our approach of immobilizing SAH via its carboxyl group and a spacer arm, he and Dr. S. K. Sharma devised another affinity system based on this principle for the purification of furanocoumarin O-methyltransferase from *Ruta graveolens* L.[42].

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