The interaction of hydralazine with a semicarbazide-sensitive amine oxidase in brown adipose tissue of the rat

Its use as a radioactive ligand for the enzyme

Margery A. BARRAND* and Brian A. CALLINGHAM

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge CB2 2QD, U.K.

Hydralazine is a potent irreversible inhibitor of the semicarbazide-sensitive amine oxidase (SSAO) found in brown fat. Initially it may act on the enzyme as a competitive inhibitor, but irreversible inhibition occurs rapidly in a concentration- and temperature-dependent manner. The presence of primary amines known to be substrates for the enzyme, but not of secondary amines, which are not metabolized by SSAO, diminishes this rate of inactivation, whereas removal of O₂ is without effect. The kinetic pattern of inactivation of SSAO by hydralazine is consistent with an active-site-directed site-saturable binding followed by the development of an irreversible enzyme-inhibitor complex. [³H]Hydralazine, used as an affinity label for SSAO, shows saturable binding to brown-fat membranes. This binding is inhibited by other inhibitors of SSAO. The rate of binding to membrane pellets containing SSAO is not affected by substrates for the enzyme. However, if solubilized partially purified SSAO preparations are used instead, the rate of binding is lowered in the presence of the SSAO substrate benzylamine. ³H-labelled material solubilized from [³H]hydralazine-treated membrane pellets by Triton X-100 at that detergent/protein ratio which releases SSAO from membranes shows the same gel-filtration characteristics as SSAO and appears by lentil lectin-agarose affinity chromatography to contain similar carbohydrate moieties. ³H-labelled material, partially purified by gel filtration and affinity chromatography, produces predominantly a single band of radioactivity on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The position of this band corresponds to an *M_r* of about 94000, almost exactly half the *M_r* already estimated for the functional unit of SSAO. Radiolabelled hydralazine may thus be used as a label for purified SSAO, but it is not specific enough to be suitable as a ligand *in vivo*.

INTRODUCTION

An amine oxidase insensitive to clorgyline, an irreversible acetylenic inhibitor of monoamine oxidase (EC 1.4.3.4), but sensitive to the carbonyl reagent semicarbazide, has been identified in various tissues of the rat, including the aorta (Coquil *et al.*, 1973; Urdin & Fuentes, 1983), heart (Clarke *et al.*, 1982), mesenteric blood vessels (Fuentes & Neff, 1977; Callingham *et al.*, 1983) and anococcygeus muscle (Callingham, 1982). This enzyme, previously referred to as clorgyline-resistant amine oxidase, is now generally known by the name semicarbazide-sensitive amine oxidase (SSAO). Its preferred substrate appears to be benzylamine, which so far has not been shown to be a physiologically important compound. The enzyme shows very low affinity for noradrenaline and no affinity for secondary or tertiary amines (Barrand & Callingham, 1982). Its molecular size and carbohydrate content shows certain similarities to the copper-containing plasma amine oxidases (Yasunobu *et al.*, 1976; Falk *et al.*, 1983). However, the idea that copper is a vital component of SSAO activity is now in doubt (Lyles *et al.*, 1983; Barrand & Callingham, 1984a). Owing to the high SSAO activity in organs rich in smooth muscle, especially vascular smooth muscle, a localization to this

particular cell type has been suggested (Lewinsohn, 1981). However, SSAO has also been identified in brown adipose tissue of the rat (Barrand & Callingham, 1982), and furthermore shown to be associated with the fat-cell itself (Barrand *et al.*, 1984). Evidence from studies both in the aorta (Wibo *et al.*, 1980) and in brown adipose tissue (Barrand & Callingham, 1982; Barrand *et al.*, 1984) strongly support the concept that SSAO resides at or in the outer cell membrane. However, the role of SSAO in the events that occur at the cell surface has yet to be determined.

It has been shown that the antihypertensive agent hydralazine is a potent irreversible inhibitor of the enzyme in the rat heart and aorta (Lyles & Callingham, 1982; Lyles *et al.*, 1983). Baker *et al.* (1985) have shown that radioactively labelled hydralazine injected into rats may be found with the elastic lamina and around the edges of smooth-muscle cells in blood vessels. The mechanisms of action of hydralazine on smooth muscle are far from clearly elucidated. Although some studies suggest that hydralazine interferes with Ca²⁺ uptake (McLean *et al.*, 1978; Lipe & Moulds, 1981), other evidence disputes this notion (Kreye & Schlicker, 1980), particularly concerning the long-term actions of hydralazine (Kwan & Daniel, 1982), and more-recent studies

Abbreviation used: SSAO, semicarbazide-sensitive amine oxidase.

* To whom correspondence should be addressed.

suggest that part of the action of hydralazine may be mediated via prostaglandins (Maekawa *et al.*, 1984). In view of the direct vasodilator action of hydralazine, the observation that it is an irreversible inhibitor of SSAO is of particular interest in connection with the possible role of SSAO at the cell surface.

The present paper describes an attempt to examine the interaction of hydralazine with SSAO in more detail and to determine whether radioactively labelled hydralazine could be employed as a suitable ligand as a probe for the enzyme and as a marker for the enzyme within the tissues. Brown adipose tissue was used as a convenient source of the enzyme, particularly since SSAO in this tissue has been well characterized (Barrand & Callingham, 1982, 1984a). Preliminary results of the work described here have already been reported (Barrand & Callingham, 1984b,c).

EXPERIMENTAL

Membrane-bound, solubilized and partially purified SSAO preparations

Male Sprague-Dawley rats, 200–400 g in weight, were killed by a blow to the head followed by decapitation, and interscapular brown adipose tissue was removed. The tissue was homogenized (1:20, w/v) in 1 mM-potassium phosphate buffer (prepared by adjusting 1 mM-KH₂PO₄ to pH 7.8 with solid KOH at 22 °C) and centrifuged in an MSE Chilspin centrifuge at 600 *g* (r_{av} , 11.3 cm) for 10 min at 5 °C to remove unbroken cells and cellular debris. The supernatant from this spin formed the 'original homogenate'.

'Membrane-bound SSAO' was pelleted from this supernatant by centrifugation in an 8 × 50 ml rotor operated at 100,000 *g* (r_{av} , 7.62 cm) or a 10 × 10 ml rotor operated at 100,000 *g* (r_{av} , 5.61 cm) in an MSE PrepSpin centrifuge for 1 h at 4 °C, and redispersed in phosphate buffer. The protein content of the various fractions was determined either by the spectrophotometric method of Lowry *et al.* (1951), with bovine serum albumin as standard, or by the fluorimetric method of Udenfriend *et al.* (1972).

'Solubilized SSAO' was prepared from the membrane pellets by treatment with Triton X-100. The protein content of the pellet samples was first adjusted to a final concentration of 1 mg/ml and then mixed at 4 °C with 0.08% (w/v) Triton X-100 in 20 mM-phosphate buffer, pH 7.8, left for 10 min before centrifuging as described above at 100,000 *g* for 1 h. The resultant supernatant formed the 'solubilized SSAO' preparation, and the resultant pellet redispersed in 1 mM-phosphate buffer was taken as the 'SSAO-poor membrane fraction'.

'Solubilized SSAO' was purified further by gel filtration and affinity chromatography (see below) to yield the 'gel filtrate' preparation and 'affinity-chromatography eluate' preparation.

Enzyme assay and inhibition studies

Amine oxidase activity was determined radiochemically by a method modified from that of Callingham & Lavery (1973) by replacement of benzene by toluene at the extraction stage. [¹⁴C]Benzylamine (sp. radioactivity 10 μ Ci/ μ mol; 25 μ M) was used as substrate for SSAO. The spectrophotometric method of Koechli & von Wartburg (1978) was used for determining SSAO activity

against non-radioactive substrates and potential substrates.

Inhibition studies were carried out either (a) by preincubating enzyme samples with inhibitor for various lengths of time between 0 and 45 min at 4 °C, at room temperature (approx. 22 °C) or at 37 °C and then diluting the samples 50-fold with 1 mM-phosphate buffer before assay for SSAO activity, or (b) by assaying for SSAO activity in the presence of inhibitor without preincubation or dilution with a maximum of 3 min incubation to minimize further interaction between enzyme and inhibitor during the assay period.

Binding studies with radiolabelled hydralazine

These were conducted on 'membrane-bound SSAO', 'SSAO-poor membrane fraction', 'gel filtrate' and 'affinity-chromatography eluate' preparations. Two different methods were employed.

(i) **Pelleting for the 'membrane-bound SSAO' and 'SSAO-poor membrane' preparations.** Samples were added to 1.5 ml polypropylene microcentrifuge tubes, together with buffer and distilled water or binding inhibitor at the appropriate concentration to give a final concentration of 6 mg of protein/ml and 20 mM-potassium phosphate buffer, pH 7.8, and incubated at 37 °C for 30 min. [³H]Hydralazine was then added to each tube to give a final concentration of 0.05–2.0 μ M and specific radioactivity of either 0.2 or 2 Ci/mmol and incubated for up to 30 min at 37 °C or at 4 °C. Binding was terminated by adding 15 μ l samples to tubes containing ice-cold 8% (w/v) trichloroacetic acid. Tubes were left on ice for 10 min, and the precipitated protein was separated from free and reversibly bound hydralazine by centrifugation in an Eppendorf 3200 microcentrifuge at 6000 *g* for 1.5 min. The protein pellets were twice soaked for 10–20 min in distilled water and re-centrifuged, and then digested with 500 μ l of Soluene-350 (Packard Instruments, Caversham, Berks., U.K.) at room temperature before washing with two 0.5 ml portions of ethoxyethanol into scintillation vials.

(ii) **Column filtration for the soluble material, i.e. 'gel filtrate' and 'affinity-chromatography eluate' preparations.** Incubation and preincubation were undertaken as above in (i), but binding was terminated and free hydralazine was separated from protein-bound hydralazine by passing 50 μ l portions of the incubation mixture down desalting columns (0.8 cm × 2 cm) of Sephadex G-50 (effective fractionation range M_r 1000–30,000; Pharmacia, Uppsala, Sweden) previously equilibrated with 0.1% Triton X-100 in 20 mM-potassium phosphate buffer, pH 7.8. Portions (350 μ l) of Triton X-100/buffer were added to wash the samples down the columns, and 400 μ l portions were used to wash out the protein-bound hydralazine fractions. These were collected into 20 ml scintillation vials, and 10 ml of Unisolve E scintillant (Koch-Light, Haverhill, Suffolk, U.K.) was added per vial. Unbound hydralazine, which was delayed on the gel, was washed out of the columns with 10 ml of Triton-buffer and discarded.

H.p.l.c.

The purity of each radioactive hydralazine batch was monitored before use. Samples (20 μ l) of [³H]hydralazine containing 1 μ Ci of tritium together with 2 μ g of unlabelled hydralazine were injected through a Rheodyne

injector loop on to a Nucleosil 10-C18 column (0.46 cm $\times$ 25 cm) previously equilibrated with 32% (v/v) methanol containing 0.5% trifluoroacetic acid, and washed through at a rate of 1 ml/min with a model 302 pump fitted with a model 802 manometric module (Gilson; Anachem, Luton, Beds., U.K.). Hydralazine was detected by absorbance at 260 nm in a Lambda-Max model 480 LC spectrophotometer (Waters, Milford, MA, U.S.A.). Tritium was detected in an Isoflo radioactivity monitor (Nuclear Enterprises, Edinburgh, U.K.) fitted with a prepacked solid scintillator cell (maximum efficiency 30% at 1.25 kV), radioactivity being monitored every 0.2 min. With this system hydralazine showed a retention time of 1 min. With [^3H]hydralazine samples, two main peaks of radioactivity were detected, the one containing 70% of the radioactivity corresponding to the position of non-radioactive hydralazine. After 2–3 months' storage in liquid N_2 , several extra peaks of radioactivity were evident. The hydralazine peak then corresponded to only about 10% of the total radioactivity. Owing to the obvious instability of the radioactive form of hydralazine, only newly opened vials were used for the binding studies.

Gel filtration

This was carried out on a column (1.6 cm $\times$ 48 cm) of Ultrogel AcA-34 (effective fractionation range M_r 20000–350000; LKB, Bromma, Sweden) with a flow rate of approx. 12 ml/h. Columns were equilibrated and material was eluted with 20 mM-phosphate buffer, pH 7.8, containing 0.1% Triton X-100. Calibration was as described previously (Barrand & Callingham, 1984a).

Affinity chromatography

This was undertaken with lectin from *Lens culinaris* immobilized on beaded agarose containing 4.5 mg of lectin/ml of gel (Sigma Chemical Co., Poole, Dorset, U.K.) as described previously (Barrand & Callingham, 1984a).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

This was performed on 2 mm-thick horizontal slab gels in an LKB 2117 Multiphor apparatus. The gels (7.5% acrylamide concentration) were prepared by degassing and filtering 1% (v/v) $\text{NNN}'\text{N}'$ -tetramethylethylenediamine, 0.4 M-sodium phosphate buffer, pH 7.2, containing 0.4% sodium dodecyl sulphate, 20% acrylamide containing 0.69% NN' -methylenebisacrylamide and water mixed in the proportions (1:2:2:2, by vol.). A further 1 vol. of 0.5% freshly prepared ammonium persulphate was added and the gel was poured. Gels were used within 24 h of preparation. Protein samples were solubilized by boiling for 10 min in 2% (w/v) sodium dodecyl sulphate and 5% (v/v) 2-mercaptoethanol in 0.4 M-sodium phosphate buffer, pH 7.2. Then 20 μl portions together with Bromophenol Blue as a marker were loaded on to 1 cm $\times$ 0.5 cm pieces of Whatman 3MM paper overlaid on the gel. Gels cooled by circulating water were run overnight at 40 mA constant current. They were fixed and stained in 0.1% Coomassie Brilliant Blue R in methanol/water/acetic acid (9:9:2, by vol.) for 1–2 h at 37 $^\circ\text{C}$, and then destained in 7.5% (v/v) acetic acid containing 5% (v/v) ethanol at 37 $^\circ\text{C}$ until the protein bands were clearly resolved. The position of radioactive material within the gels was determined by

eluting 0.5 cm strips overnight at 30 $^\circ\text{C}$ in 1 ml of Soluene before addition of scintillant. By this method, recovery of radioactive material applied to the gel was over 70%. Each gel was calibrated with standard proteins (ovalbumin, phosphorylase *a* and bovine serum albumin, the M_r values of these being taken as 43000, 93000 and 67000 respectively) and a mixture of cross-linked bovine haemoglobin (M_r values of 16000 for monomeric, 32000 for dimeric, 48000 for trimeric and 64000 for tetrameric forms of haemoglobin respectively).

Materials

[$\text{G-}^3\text{H}$]Hydralazine hydrochloride was kindly synthesized and donated to us by Dr. D. E. Brundish and Dr. P. D. Kane of Ciba Laboratories, Horsham, Sussex, U.K. [$1\text{-}^{14}\text{C}$]Benzylamine hydrochloride was purchased from Amersham International (Amersham, Bucks., U.K.). All other compounds were either standard laboratory reagents of analytical grade or were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

Inhibition by hydralazine of 'membrane-bound SSAO' activity was found to take place at a rate dependent both on temperature and on the concentration of inhibitor (Fig 1), suggestive of pseudo-first-order kinetics (Rando, 1984). The inactivation curves produced by preincubation with inhibitor at 37 $^\circ\text{C}$ followed by dilution appeared to flatten off with time, making it difficult to calculate initial rates. More-linear relationships were seen if preincubation was at 4 $^\circ\text{C}$. Extra enzyme added after 10 min preincubation at 37 $^\circ\text{C}$ was only slightly or not at all inactivated, suggesting that the flattening off in the inactivation curve was due to loss of available hydralazine either by decomposition or due to binding to other components in the membrane pellets (Waley, 1980). When inhibition of SSAO activity was studied without preincubation or dilution [see method (b) in the Experimental section], in the range of hydralazine concentrations between 0.05–0.2 μM , curves parallel to that of the control, consistent with competitive inhibition, were produced when a plot of $[\text{S}]/v$ against $[\text{S}]$ was constructed. From such plots apparent K_i values in the region of 0.2–0.3 μM were calculated. However, the rate of conversion into the irreversible complex is probably too rapid to be able to obtain reliable values for kinetic parameters from these experiments.

The rate of inactivation of the enzyme was slowed by the presence of primary amines known to be substrates for SSAO, the substrates with the highest affinity for the enzyme having the greatest protective effect (Figs. 2a and 2b). Diamines and polyamines, which are not metabolized by SSAO, were without effect (Figs. 2c and 2d). Occupation of the active site of the enzyme therefore interferes with the inhibitor–enzyme interaction, suggesting that hydralazine causes site-directed inactivation.

No evidence could be found to indicate that hydralazine acts initially as a substrate. Excluding O_2 from the reaction by bubbling N_2 through the medium before and during the preincubation phase did not alter the rates of inactivation of SSAO by 1 μM -, 0.5 μM - and 0.1 μM -hydralazine at room temperature. Molecular O_2 is required for the deamination of the monoamine substrates by SSAO (Clarke *et al.*, 1982), but clearly the interaction with hydralazine is not dependent on the

Fig. 1. Inactivation of 'membrane-bound SSAO' by various concentrations of hydralazine at 37 °C and at 4 °C

'Membrane-bound SSAO' samples containing 6 mg of protein/ml were incubated in 20 mM-potassium phosphate buffer, pH 7.8, alone (○), or in buffer with 0.2 μM - (△), 0.4 μM - (▲), 0.6 μM - (▽) or 0.8 μM - (▼) hydralazine at (a) 4 °C or (b) 37 °C. Samples (15 μl) were taken after intervals of time between 0 and 45 min and each was diluted with 750 μl of ice-cold water. SSAO activity remaining in these diluted samples was assayed in quadruplicate and compared with activity remaining in control diluted samples not preincubated. Results from one of four separate experiments are shown. At higher hydralazine concentrations, i.e. 1 μM and above, SSAO activity was completely abolished.

Fig. 2. Effect of certain amines on the rate of inactivation of 'membrane-bound SSAO' by hydralazine

'Membrane-bound SSAO' samples containing 6 mg of protein/ml were preincubated at 37 °C in 20 mM-potassium phosphate buffer, pH 7.8, alone (○), in buffer with 0.4 μM - (a and d) or 0.6 μM - (b and c) hydralazine (△), in buffer with hydralazine in the presence of 100 μM -benzylamine (●), 100 μM -2-phenethylamine (□) or 100 μM -tyramine (■), i.e. substrates for SSAO with K_m values approx. 2 μM , 10 μM and 40 μM respectively (Barrand & Callingham, 1982), or in buffer with hydralazine in the presence of 100 μM -histamine (◇) or 100 μM -spermidine (◆), i.e. amines not substrates for SSAO. Samples (15 μl) were taken after 0, 5, 10 and 20 min and each was diluted with 750 μl of ice-cold water. SSAO activity remaining in these diluted samples was assayed in quadruplicate and compared with control samples not preincubated. Results from one of three separate experiments are shown.

presence of O_2 . The SSAO–hydralazine interaction is thus unlike that seen between hydrazines and monoamine oxidase, where O_2 appears to be essential for the irreversible step (Kenney *et al.*, 1979). The irreversible interactions of hydrazines with the non-flavin plasma amine oxidase still takes place under anaerobic conditions (Massey & Churchich, 1977; Lindström & Pettersson, 1978). In addition, when samples of ‘membrane-bound SSAO’ were investigated by the Koechli & von Wartburg (1978) method, which measures H_2O_2 production, no generation of peroxide could be detected when hydralazine at concentrations between 0.1 and $10 \mu M$ was added as a potential substrate. This photometric method has been used to assay monoamine oxidase with a number of non-radioactive substrates (Peers *et al.*, 1980), including benzylamine. When it was used to assay SSAO the K_m values obtained were similar to those from the radiochemical method (M. A. Barrand & B. A. Callingham, unpublished work). SSAO, like monoamine oxidase, therefore generates H_2O_2 as one of the products of metabolism. It is likely, however, that hydralazine itself influenced the assay. In the absence of the membrane sample, hydralazine itself caused a change in absorbance, the rate of change being proportional to the concentration of hydralazine used. Subsequent addition of membranes to the mixture decreased this rate of change in absorbance, presumably by binding the free hydralazine. It has been shown that hydralazine produces free-radical intermediates during oxidation catalysed by metals (Sinha & Patterson, 1983).

The suitability of hydralazine as an affinity label for SSAO was investigated with [3H]hydralazine. Binding of [3H]hydralazine showed a similar time course to SSAO inactivation in the same membrane preparations and was both temperature- and concentration-dependent. At concentrations between 0.5 and $1 \mu M$, binding of [3H]hydralazine to ‘membrane-bound SSAO’ preparations was decreased by a similar extent after preincubation of the membrane samples with either $10 \mu M$ -hydralazine, $10 \mu M$ -phenelzine or $10 \mu M$ -*O*-benzylhydroxylamine, all three potent irreversible inhibitors of SSAO (Barrand & Callingham, 1982). Benzylhydroxylamine ($10 \mu M$) was used in all subsequent binding studies to define the extent of non-specific binding. ‘Specific’ binding of [3H]hydralazine to ‘membrane-bound SSAO’ preparations containing 6 mg of protein/ml was found to be saturable, reaching a plateau at about $1 \mu M$ -hydralazine concentration.

It is most unlikely that the entire [3H]hydralazine binding to membrane fractions represented binding to SSAO alone. Although saturation of binding was evident, the rate of binding of $0.5 \mu M$ -[3H]hydralazine to ‘membrane-bound SSAO’ preparations measured at $4^\circ C$ was unaltered by the presence of the SSAO substrate benzylamine. When samples of membrane pellets, pretreated with [3H]hydralazine at $1 \mu M$, were treated with various concentrations of Triton X-100 (Fig. 3), release of material into the supernatant occurred within the same range of detergent/protein ratios, i.e. 0.3–1.2:1, that are known to release SSAO from membranes (Barrand & Callingham, 1984a). But, whereas 80–90% of the total SSAO activity was identifiable in the soluble fraction at a Triton/protein ratio of 1.2:1, only 40% of the total tritiated material appeared in the supernatant. A large proportion of radioactivity remained bound to the pellet. Furthermore there was a significant amount of ‘specific’

Fig. 3. Solubilization of tritium-labelled material from [3H]hydralazine-treated brown-adipose-tissue membrane fractions by titration with Triton X-100

Samples of membrane pellets containing 14 mg of protein/ml were incubated at $37^\circ C$ with $0.1 \mu M$ -[3H]hydralazine (20 Ci/mmol) for 30 min and twice washed with 20 mM-potassium phosphate buffer, pH 7.8, and centrifuged at $100\,000 g$ for 1 h to remove unbound [3H]hydralazine. After adjustment to 1 mg of protein/ml, samples of pellet were then treated at $4^\circ C$ with 0.01%, 0.02%, 0.04%, 0.08%, 0.12%, 0.16% and 0.2% (w/v) Triton X-100 in the phosphate buffer and centrifuged again at $100\,000 g$ for 1 h. The tritium content of supernatant (○) and pellet (●) samples was then measured in quadruplicate. Each point is the mean $\pm$ S.E.M. of six values obtained from three separate experiments.

binding of [3H]hydralazine to the residual membrane pellets remaining after removal of most of the SSAO by Triton X-100. Maximum binding to ‘original homogenates’ was about 6 pmol/mg of protein, to ‘membrane-bound SSAO’ preparations about 65 pmol/mg of protein, and to ‘SSAO-poor membrane’ preparations about 46 pmol/mg of protein. Clearly, hydralazine is also binding avidly to sites in the membrane other than SSAO.

When the solubilized tritiated material was washed through an Ultrogel Aca-34 column, most of the radioactivity showed an elution profile similar to that of SSAO activity (Fig. 4). Pooled concentrated tritium-enriched fractions from the gel column were washed on to a column of *Lens culinaris* lectin–agarose and eluted with 0.25 M- α -methyl D-mannoside. About 70% of the radioactivity applied was retained on the gel and subsequently displaced by the mannoside (Fig. 5) in a manner similar to that of the solubilized SSAO. These similarities in gel-filtration and affinity-chromatography characteristics indicate that the solubilized [3H]hydralazine-bound material is of a similar molecular size to SSAO and contains similar carbohydrate moieties. Furthermore the rate of binding of [3H]hydralazine to the partially purified SSAO, i.e. ‘gel filtrate’ or ‘affinity-chromatography eluate’ preparations, was diminished in the presence of the SSAO-specific substrate benzylamine (Fig. 6), suggesting that a significant amount of radiolabelling in these preparations takes place at the active site of the enzyme.

Tritium-enriched fractions eluted from the affinity column and concentrated to 0.1 mg of protein/ml were subjected to denaturation followed by sodium dodecyl

Fig. 4. Gel-filtration profiles of SSAO and of tritium-labelled material solubilized from brown-adipose-tissue membranes

(a) SSAO solubilized with 0.08% Triton X-100 was loaded on to a calibrated column of Ultrogel AcA-34 and eluted with 20 mM-phosphate buffer, pH 7.8, containing 0.1% Triton X-100. Each fraction (0.96 ml) was assayed in quadruplicate for SSAO activity (○) with 25 μ M-[14 C]benzylamine as substrate and for protein (A_{750} ; ▲) by the method of Lowry *et al.* (1951). Total enzyme activity recovered was over 80% of that applied to the column. (b) Tritium-labelled material solubilized with 0.08% Triton X-100 from [3 H]hydralazine-treated membrane fractions was loaded on to an Ultrogel AcA-34 column and eluted as in (a). Each fraction was measured in quadruplicate for its tritium content (●) and for protein (A_{750} ; ▲). Total radioactivity recovered was over 90% of that applied to the column. Results shown are from one of four different preparations investigated. Arrows indicate position of void volume. The bed volume (not shown) was at fraction 90.

sulphate/polyacrylamide-gel electrophoresis. Some 90% of the radioactivity recovered from the gel was concentrated in a single peak at a position corresponding to an M_r of 94 000 (Fig. 7). This is almost exactly half the M_r of the functional unit of SSAO as judged both by gel filtration and by radiation-inactivation analysis (Barrand & Callingham, 1984a). If indeed this peak really does represent radiolabelled denatured SSAO, it would seem to indicate that the functional enzyme is made up of subunits at least one of which is half the size of the enzyme and contains the active centre. It is interesting to note the similarities with pig plasma amine oxidase. This enzyme also has been estimated to have an M_r of between 186 000 and 196 000 (Falk, 1983) and in the presence of denaturing reagents to behave as a single species of M_r 95 000–97 000 (Barker *et al.*, 1979). Whether these two subunits are chemically distinct is not known, although there is some evidence for identical primary structure (Barker *et al.*, 1979). The stoichiometry of enzyme inactivation by phenylhydrazine, which, like hydralazine inactivation of SSAO, interacts at the active site of the enzyme (Lindström *et al.*, 1974), is interesting in that it shows only 1 mol of hydrazine/mol of the dimeric enzyme. The question therefore arises as to how two apparently identical subunits contain only one active centre. Suggestions of negative co-operativity, purification inactivation of one subunit or action of the enzyme via a flip-flop mechanism with only one subunit active at a time have been put forward, but as yet the problem has

not been resolved (Falk, 1983). On the basis of the carbohydrate content also, SSAO shows definite similarities to the plasma amine oxidases (Falk *et al.*, 1983). On the other hand, SSAO is a membrane-bound enzyme, unlike the plasma enzymes, and, although not inactivated by solubilization, cannot remain in the soluble form without detergent. It also shows differences in substrate specificity (Barrand & Callingham, 1982) and appears to be relatively insensitive to Cu^{2+} -chelating agents, although this may be due to an inaccessibility of the metal. If indeed SSAO is closely related to the plasma enzymes, then maybe these differences are due to alterations in tertiary structure rather than in the fundamental subunits.

Here it has been shown that hydralazine is not very specific for SSAO and binds well at other sites. Hydralazine is already known to affect a variety of enzymes. On some it acts in a reversible fashion as a chelator and no specific binding occurs, e.g. prolyl hydroxylase (Bhatnagar *et al.*, 1972) and dopamine- β -hydroxylase (Liu *et al.*, 1974). With lysyl oxidase, inhibition is irreversible and enzyme activity cannot be restored by dialysis in the presence of either the putative cofactor pyridoxal or Cu^{2+} (Numata *et al.*, 1981). When radiolabelled hydralazine (Baker *et al.*, 1985) was given intravenously to rats, persistent labelling of blood vessels, particularly arteries, was seen in whole-body autoradiographs, the silver grains being deposited over the elastic laminae and around the smooth-muscle cells. This distribution was thought to represent binding of

Fig. 5. Affinity-chromatography profile of solubilized SSAO and of solubilized tritium-labelled material on a column of *Lens culinaris* lectin-agarose

(a) Solubilized SSAO, partially purified by gel filtration, was loaded on to a column of *Lens culinaris* lectin immobilized on agarose beads, equilibrated with 20 mM-phosphate buffer, pH 7.8, containing 0.1% Triton X-100, 0.1 mM-MgCl₂, 0.1 mM-CaCl₂, 0.1 mM-MnCl₂ and 0.5 M-NaCl and washed with 8 column volumes of the buffer (fractions 1–8). Then 2 column volumes of 20 mM-phosphate buffer/Triton containing 0.01 M-EDTA were passed down the column (fractions 9–10) followed by a continuous gradient from 0 to 0.5 M- α -methyl D-mannoside (---) in the buffer/Triton (fractions 11–45). Samples (0.5 ml) of the eluate from each fraction were collected and assayed in quadruplicate for SSAO activity (○) with 25 μM -[¹⁴C]benzylamine as substrate and for protein (A_{750} ; ▲) by the method of Lowry *et al.* (1951). Over 50% of the enzyme activity and over 85% of the protein applied was recovered. (b) Soluble tritium-labelled material obtained from the tritium-enriched gel-filtration fractions was loaded on to the affinity column and eluted as in (a). The tritium content (●) and protein content (A_{750} ; ▲) of each fraction were measured in quadruplicate. Over 80% of the radioactivity applied to the column was recovered.

Fig. 6. Effect of benzylamine on the rate of binding of [³H]hydralazine to 'gel filtrate' preparations

Samples of 'gel filtrate' containing between 0.3 and 0.8 mg of protein/ml were preincubated at 37 °C for 30 min with (△) or without (□ and ○) 10 μM -O-benzylhydroxylamine. They were then incubated at 4 °C with 0.1 μM -[³H]hydralazine (2 Ci/mmol) in the presence (□) or absence (○ and △) of 100 μM -benzylamine. At 0, 4, 8 and 12 min 50 μl samples were applied to desalting columns of Sephadex

radiolabelled hydralazine to lysyl oxidase in the laminae and to SSAO around the smooth-muscle cells. Since hydralazine binds to the active site of SSAO, it is possible that clues to the disposition of its active site within the cell membrane, i.e. the sidedness of the enzyme, might be obtained from such autoradiography. However, the resolution of the technique was insufficient to show whether the silver grains were intra- or extra-cellular. To resolve the question of the sidedness of the active site of SSAO, it is clear that other techniques, e.g. the use of site-specific agents unable to cross the cell membrane, would be preferable.

In conclusion, [³H]hydralazine has proved useful in characterizing the subunit of SSAO, so revealing similarities to the plasma amine oxidases. Whether it can be used as a probe for the active site of the enzyme in the way that borohydride-reduced radioactive benzylamine has been employed to explore the structure of bovine plasma amine oxidase (Inamasu *et al.*, 1974) remains to be seen. But it is clearly not stable or specific enough to

G-50 to separate protein-bound from free hydralazine. The radioactivity associated with the protein appearing in the void volume was measured. Each point is the mean $\pm$ S.E.M. of six values obtained from duplicate determinations in three experiments done on three separate preparations, normalized to d.p.m./μg of protein applied to the column.

Fig. 7. Gel-electrophoresis profile of partially purified tritium-labelled material solubilized from [^3H]hydralazine-treated brown-adipose-tissue membranes

(a) Solubilized tritium-labelled material partially purified by gel filtration and affinity chromatography was concentrated to 0.1 mg of protein/ml, then denatured by boiling in 2% sodium dodecyl sulphate for 10 min, and 20 μl portions were applied to a polyacrylamide gel along with 20 μl portions of standard proteins. The water-cooled gel was run overnight at 40 mA constant current and then stained for protein with 0.1% Coomassie Blue. The positions of the protein bands measured within the gel are shown in diagrammatic form. Strip (i) represents phosphorylase *a* (M_r 93000), strip (ii) cross-linked haemoglobins (M_r 16000, 32000, 48000 and 64000 for monomer, dimer, trimer and tetramer respectively) and strip (iii) the tritium-labelled material. (b) The position of radioactive material within the gel was then determined by eluting 0.5 cm strips of gel overnight at 30 $^{\circ}\text{C}$ in 1 ml of Soluene before addition of scintillant. The result shown here is from one of six different experiments performed.

be used for active-site titration or for identification of the disposition of the enzyme within whole tissue.

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