

Purification and characterization of membrane-bound semicarbazide-sensitive amine oxidase (SSAO) from bovine lung

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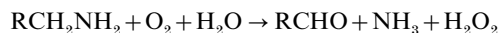
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Semicarbazide-sensitive amine oxidase (SSAO) has been purified from bovine lung microsomes in a form which is catalytically active and stable to storage. The enzyme, an integral membrane protein, was solubilized with Triton X-100 and purification was achieved, in the presence of detergent, by chromatography with Cibacron Blue 3GA-agarose, hydroxylapatite, *Lens culinaris*-agarose, Resource Q-FPLC and gel filtration on Superdex 200 HR-FPLC. This is the first reported procedure for the extensive purification of a membrane-bound SSAO. The purified enzyme had an apparent M_r of 400 000 but exhibited microheterogeneity with SDS/PAGE and isoelectric focusing, probably as a result of its glycoprotein nature. It behaved as a tetramer with subunits with apparent M_r values of 100. Antibodies raised towards the purified enzyme cross-reacted with the enzymes from human lung

and bovine plasma. Redox-cycling staining and reaction with carbonyl reagents were consistent with the presence of a quinone cofactor, possibly topa quinone. The enzyme was also shown to contain two mol of Cu/mol of enzyme and removal of half of this bound copper resulted essentially in complete inhibition of enzyme activity. In contrast to the reported behaviour of the SSAO enzymes from plasma, the bovine lung enzyme was relatively insensitive to inhibition by cyanide, copper-chelating agents and amiloride. The specificity of the bovine lung enzyme was also narrower than reported for soluble SSAO. It catalysed the oxidative deamination of benzylamine, methylamine, 2-phenylethylamine and histamine but had no significant activity towards dopamine, 5-hydroxytryptamine, tryptamine or tyramine.

INTRODUCTION

Semicarbazide-sensitive amine oxidases (SSAO) are a diverse group of enzymes within the classification EC 1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper-containing)]. They include beef and sheep plasma spermine oxidase, plasma amine oxidase, diamine oxidase and a tissue-bound SSAO enzyme. All of them are inhibited by semicarbazide, as a result of the presence of a carbonyl group at the cofactor site, and this is frequently used to distinguish these enzymes from the monoamine oxidases (MAOs), EC 1.4.3.4 [amine:oxygen oxidoreductase (deaminating) (flavin containing)]. However the substrate specificities of both types of enzyme overlap to some extent. The oxidation of aromatic and aliphatic primary amines by SSAO enzymes produces the corresponding aldehyde, ammonia and hydrogen peroxide:



The non-physiological amine benzylamine has been widely used as the substrate in studies of SSAO enzymes from plasma and tissues from several species. Soluble plasma SSAOs from several species have been purified successfully and have been shown to contain, per mol of enzyme, 2 mol of tightly-bound copper and 2 mol of the uncommon covalently-bound organic cofactor, topa quinone (the quinone formed from the oxidation of 2,4,5-trihydroxyphenylalanine), integrated into the polypeptide [1,2].

The membrane-bound SSAO has been found in several tissues, with particularly high activity in blood vessels associated with smooth-muscle cells [3,4]. A secreted soluble form of SSAO from

cultured vascular smooth-muscle cells has also been described [5]. SSAO activity has also been found in some other non-vascular cell types such as chondrocytes in rat articular cartilage [6], adipocytes from rat white and brown fat [7,8], in pig dental pulp [9] and in different parts of the bovine eye [10]. In spite of its wide tissue distribution, its physiological role is still far from clear. However, its presence in plasma membrane from vascular smooth-muscle might suggest a role in metabolism of circulating amines. MAO activities have been characterized in perfused preparations of lung, and this tissue has been shown to be potentially important in amine metabolism [11,12]. SSAO present in the microsomal fraction from human and bovine lung [13] may be important in the metabolism of volatile amines since it is active towards short-chain aliphatic amines [14,15], whereas MAO is not.

SSAO activity appears to be altered in some pathological conditions. Lewinsohn [3] described changes in plasma SSAO activity in fibrotic liver and patients suffering from burns. A decreased membrane-bound SSAO activity has been reported in chemically-induced rat breast tumours [16]. On the other hand, formaldehyde generation by SSAO in vascular tissues could be a potential risk factor for stress-related angiopathy [17]. It has been reported recently that SSAO is associated with the glucose transporter GLUT4 from adipocyte membrane [18], supporting the previous finding that raised plasma SSAO levels in rats with streptozotocin-induced diabetes mellitus could be prevented by insulin replacement therapy [19].

Although soluble SSAO from plasma of several species has been purified to apparent homogeneity (see for example [20]), there have been few reported attempts at purification and characterization of the tissue-bound enzyme. Recently the se-

quence of a 4040 bp cDNA, cloned from a human placental library, for a topa-quinone containing amine oxidase has been reported [21]. This sequence showed 84% identity with the cDNA for some serum amine oxidases. The absence of domains that would interact with membranes raised the possibility that this might also correspond to a soluble, rather than a membrane-bound SSAO. The present work was undertaken to solubilize and purify microsomal SSAO from bovine lung to allow studies on its structure, behaviour and relationships with the plasma enzymes.

EXPERIMENTAL

Materials

Acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulphate, *N,N,N',N'*-tetramethylethylenediamine (TEMED), SDS, high molecular-mass standard proteins for SDS gels, 0.45- μ m pore-size nitrocellulose membranes, Chelex 100 resin and Bio-gel HTP (a beaded form of hydroxylapatite) were obtained from Bio-Rad. Sephacryl S-300 HR, concanavalin A-Sepharose 4B, precast 3.5–9.5 ampholine polyacrylamide gel plates, isoelectric focusing standards (pI range 3.5–9.3) and pre-packed columns of Superdex 200 HR, Resource Q and Mono P were purchased from Pharmacia. Tween 20, α -methyl-D-mannopyranoside, sodium cholate, sulphosalicylic acid and phenazine methosulphate were obtained from Fluka. Centricon-30 miniconcentrators and XM-50 ultrafiltration membranes were purchased from Amicon (Danvers, MA, U.S.A.). *N*-glucosidase F, *O*-glucosidase and neuraminidase were from Boehringer-Mannheim. The radioactive substrates [¹⁴C]benzylamine hydrochloride (specific activity 55 mCi/mmol) and [¹⁴C]methylamine (779 μ Ci/mg) were obtained from Amersham International and [ethyl-1-¹⁴C]2-phenylethylamine hydrochloride (50 mCi/mmol) was from New England Nuclear. All other reagents were from Sigma. Clorgyline hydrochloride was a gift from Rhône Poulenc, formerly May and Baker Ltd. (Dagenham, U.K.).

Enzyme assays

SSAO activity was determined radiochemically by the method of Fowler and Tipton [22] with 20 μ M [¹⁴C]benzylamine (3 mCi/mmol) or 100 μ M [¹⁴C]2-phenylethylamine (2.5 mCi/mmol) as substrates. When necessary, MAO activity was inhibited by preincubating with 1 mM clorgyline for 30 min at 37 °C. The reaction was carried out at 37 °C in a final volume of 225 μ l of 50 mM potassium phosphate buffer, pH 7.2, and was stopped by the addition of 100 μ l of 2 M citric acid. Radioactively labelled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole (PPO) before liquid scintillation counting.

SSAO activity towards 100 μ M [¹⁴C]methylamine (1 mCi/mmol) as substrate was determined as described by Precious et al. [23]. The reaction was carried out at 37 °C in a final volume of 100 μ l of 50 mM potassium phosphate buffer, pH 7.2, and was stopped by cooling the tubes in an ice-bath. The reaction product ([¹⁴C]formaldehyde) was separated from any unchanged amine by the use of a 1 ml Amberlite C6-50 (carboxylic form) column, and 6 ml of Bray solution was added to the column eluate before liquid scintillation counting.

Time-course assays were used to ensure that initial rates of the reaction were determined and proportionality to enzyme concentration was established in each case. The kinetic parameters were calculated by use of non-linear regression analysis using the computer program Enzfitter (Biosoft, Elsevier, Cambridge, U.K.).

Concentrations of inhibitor that gave 50% inhibition (IC_{50} values) for some hydrazine derivatives were determined without enzyme-inhibitor preincubation, by the addition of the enzyme to the assay mixture containing substrate and inhibitor. Semicarbazide was also preincubated with the enzyme for 30 min at 37 °C before the addition of the substrate. The concentration ranges of the inhibitors used were as follows: 5×10^{-2} – 10^{-6} M for amiloride, 10^{-3} – 10^{-7} M for semicarbazide, 10^{-6} – 10^{-10} M for phenelzine (2-phenylethylhydrazine) and 10^{-7} – 10^{-10} M for phenylhydrazine. In studies on the inhibition by cyanide, the enzyme preparations were incubated with a series of concentrations of KCN (10^{-7} – 10^{-1} M) for 15 min at 37 °C before the activity was determined. The SSAO activity remaining was determined radiochemically with 20 μ M benzylamine as substrate.

Protein was measured by the method of Markwell et al. [24], with BSA as standard.

Preparation of bovine lung microsomes

Bovine lung was obtained from a local abattoir and transported immediately to the laboratory packed in ice. After removing the connective tissue, the lung was weighed, chopped into small pieces with scissors and washed extensively with saline [0.9% (w/v) NaCl] to eliminate blood as a potential source of contaminating plasma amine oxidase. The tissue was then homogenized 1:10 (w/v) in 10 mM Tris/HCl buffer, pH 7.2, containing 0.25 M sucrose, in a Waring blender, and was filtered through two layers of cheesecloth. The homogenate was subjected to differential centrifugation and the microsomal fraction was obtained by adding 10 mM CaCl₂ to the post-mitochondrial supernatant and centrifugation as described previously [15]. The final microsomal pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.2, the protein concentration was adjusted to 10 mg/ml and samples were stored in aliquots at –20 °C until required. The resulting preparation was referred as crude microsomes.

Solubilization studies

Samples of crude microsomes were mixed with equal volumes of different detergents prepared in 20 mM potassium phosphate buffer, pH 7.2, at 4 °C. The detergents and final concentrations tested were 0.2, 0.4, 0.6, 0.8 and 1.0% (w/v) Triton X-100; 0.25, 0.5, 0.75, 1.0 and 1.25% (w/v) sodium cholate and 0.25, 0.5, 0.75, 1.0 and 1.5% (w/v) β -octyl-glucopyranoside. After stirring at 4 °C for 30–60 min, the samples were centrifuged at 105 000 g for 1 h. The resulting pellets and supernatants were collected and the protein content and SSAO activity were determined for each fraction.

Purification of bovine lung microsomal SSAO

All procedures were carried out as 4 °C, unless otherwise stated. All buffers and solutions used during the FPLC chromatographic steps were filtered and degassed. The starting material, 150 ml of the crude microsomal fraction (1500 mg protein), was mixed with an equal vol. of 1.2% (w/v) Triton X-100 in 20 mM potassium phosphate buffer, pH 7.2, and the mixture was stirred for 30 min. The solubilized enzyme was obtained by decanting the supernatant after centrifugation at 105 000 g for 1 h.

The solubilized enzyme was loaded on to a Cibacron Blue 3GA-agarose column (75 ml), previously equilibrated with 0.1% (w/v) Triton X-100 in 20 mM potassium phosphate buffer, pH 7.2 (buffer A). The resin was washed with the equilibration

buffer and, after passing 100 ml of 0.5 M KCl in buffer A, the SSAO activity was eluted with 500 ml of 1 M KCl in buffer A. Fractions (10 ml) were collected and the protein content and enzyme activity were determined in each. Fractions containing SSAO activity were pooled and concentrated in an Amicon ultrafiltration cell equipped with a X-50 membrane. The concentrated enzyme was dialyzed overnight against two changes of 5 litres of 30 mM potassium phosphate buffer, pH 7.2, containing 0.1% (w/v) Triton X-100 (buffer B). The dialyzed protein was applied to a 60-ml column of hydroxylapatite Bio-gel HTP that had been equilibrated previously with buffer B. The column was then washed with 150 ml of buffer B, followed by 150 ml of 0.4 M potassium phosphate buffer, pH 7.2, containing 0.1% (w/v) Triton X-100. Fractions (10 ml) were collected and those containing SSAO activity were pooled and loaded on to a 5-ml column of *Lens culinaris*-agarose equilibrated previously with buffer A containing 0.1 mM MnCl₂, 0.1 mM CaCl₂, 0.1 mM MgCl₂ and 0.5 M NaCl (buffer C). Enzyme activity was retained and elution was achieved initially with 15 ml of 10 mM EDTA in buffer A, followed by 100 ml of a linear gradient from 0 to 1 M α -methyl-D-mannopyranoside in buffer A. Fractions (5 ml) containing SSAO activity were pooled, concentrated in an Amicon ultrafiltration cell and dialyzed overnight with two changes of 3 litres of buffer A.

The concentrated enzyme (10 ml) was applied on to a 1 ml Resource-Q column connected to an FPLC system, previously equilibrated with buffer A, at a flow rate of 1 ml/min at room temperature. After washing the column with buffer A, SSAO was eluted with 20 ml of a linear gradient (0–0.3 M NaCl) in buffer A. Fractions (1 ml) were collected on ice and those containing SSAO activity were combined and concentrated in a Centricon-30 ultrafiltration unit to a final volume of 0.5 ml. Finally, this concentrate was applied to a Superdex 200 HR column connected to the FPLC system that was equilibrated with buffer A at room temperature and calibrated with the following molecular-mass (kDa) markers: Blue Dextran (void volume), bovine thyroglobulin (669), horse spleen apoferritin (443), yeast alcohol dehydrogenase (150), bovine serum albumin (66), ovalbumin (45) and erythrocyte carbonic anhydrase (29). The column was eluted at a flow rate of 0.3 ml/min and 0.5 ml fractions were collected on ice. The active fractions were combined and concentrated in a Centricon-30 ultrafiltration unit to a final volume of 1 ml. The purified enzyme was divided into aliquots and stored at -80°C .

Electrophoresis and redox-cycling staining

All PAGE was performed with a Bio-Rad Mini Protean II apparatus. SDS 7.5% polyacrylamide gels were prepared as described by Laemmli [25]. Enzyme samples were boiled for 3 min in the presence of 5% (v/v) 2-mercaptoethanol before application on to the gels. After electrophoresis, protein bands were silver-stained (Bio-Rad). Standard proteins for calibration (kDa) were: myosin heavy chain (200), *Escherichia coli* β -galactosidase (116), phosphorylase b (97), BSA (66), glutamic dehydrogenase (55), lactate dehydrogenase (36), carbonic anhydrase (31), trypsin inhibitor (21), lysozyme (14) and aprotinin (6).

Non-denaturing electrophoresis was performed in discontinuous polyacrylamide gels, using 3% and 5% as the stacking and resolving acrylamide concentrations respectively. Gels and running buffers contained Tris/glycine, pH 8.5, with 0.1% (w/v) Triton X-100. A 15 mA constant current was applied for 75 min. Protein was silver stained or stained for SSAO activity by the

method of Falk et al. [26], using 0.25 mM benzylamine as substrate.

Isoelectric focusing was performed with a ready-prepared ampholine polyacrylamide gel plate, pH range 3.5–9.5, on a cooled Multiphor II system (Pharmacia), to which a fixed voltage of 1500 V was applied until the colour marker (Methyl Red, pI = 3.8) reached to the anode (2 h, approx.). The gel was then stained for SSAO activity or for protein. Protein staining was achieved by fixing the gel with 10% (w/v) trichloroacetic acid containing 5% (w/v) sulphosalicylic acid for 1 h, staining for 10 min with 0.025% (w/v) Coomassie Blue R 250 dissolved in methanol:acetic acid:water (40:10:50, by vol.), previously warmed to 60°C . Destaining was accomplished by several applications of the same solvent.

For redox-cycling staining, proteins separated by SDS/PAGE (7.5% gel) under reducing conditions were electroblotted on to a $0.45\ \mu\text{m}$ nitrocellulose membrane in ice-cold transfer buffer (25 mM Tris/192 mM glycine containing 20% (v/v) methanol, pH 8.3) at 50 mA constant current for 90 min. The blot was then stained with 0.24 mM Nitro Blue Tetrazolium in 0.1 M potassium glycine, pH 10, for 30–45 min [27].

Chemical cleavage of SSAO subunits was accomplished as follows. Pure SSAO (200 μg) was resolved by SDS/PAGE (7.5% gels) and electroblotted on to a cationic PVDF-based membrane (Immobilon-CD, Millipore Corp. Bedford, MA, U.S.A.). Protein was visualized by use of a negative stain (Immobilon-CD stain, Millipore), following the manufacturer's instructions. The 100, 130 and 170 kDa bands were excised from the membrane with a clean scalpel, cut into pieces of 1 mm² and eluted with 500 μl of 70% (w/v) formic acid containing 1% (v/v) Triton X-100. After cleavage with 1 mg CNBr, the resulting peptides were eluted, resolved by SDS/PAGE (15% gel) and stained with Coomassie Blue R250.

Preparation of antibodies

Preimmune serum was collected from 2 female New Zealand white rabbits. Purified SSAO from bovine lung (50 μg) was mixed with an equal volume of complete Freund's adjuvant and injected at multiple sites, both intramuscularly and intradermally, in each rabbit. After 3 weeks, each animal was boosted with 50 μg of purified enzyme in incomplete Freund's adjuvant by multiple injections, both intramuscular and intradermally. Finally, the animals received 100 μg of antigen in incomplete Freund's adjuvant. Rabbits were bled from the marginal ear vein 1, 2, 3 and 4 weeks after the last immunization and serum was separated and stored at -20°C . Anti-serum against pig plasma amine oxidase [28] was a gift from Dr. F. Buffoni (University of Florence, Italy).

Titration of the antiserum was performed by ELISA, as described elsewhere [29], using horseradish peroxidase-conjugated goat anti-rabbit IgG as the second antibody and 3-dimethylamino benzoic acid, 3-methyl-2-benzothiazoline hydrazone and H₂O₂ as substrates. The titre was defined as the dilution of antibody that gave 50% of the maximum absorbance change.

Western blotting

Proteins separated by SDS/PAGE (7.5% gel) were electroblotted on to $0.45\ \mu\text{m}$ nitrocellulose membranes as described above. After blocking, the membrane was incubated for 2 h at room temperature with the corresponding antiserum, diluted in 10 mM Tris/HCl, pH 7.2, containing 150 mM NaCl and 0.05% (w/v)

Tween 20, followed by incubation for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG. The bound antibody was detected by incubation with a solution containing 5-bromo-4-chloroindolyl phosphate and Nitro-Blue Tetrazolium in 0.1 M sodium bicarbonate 1 mM MgCl₂, pH 9.5. After colour development, the membrane was washed with distilled water, air-dried and photographed. The pre-stained molecular-mass (kDa) standards used were myosin (205), BSA (80), glutamate dehydrogenase (64), alcohol dehydrogenase (50) and carbonic anhydrase (36) (Novex, San Diego, CA, U.S.A.) or myosin (208), β -galactosidase (118), BSA (85) and ovalbumin (47) (Bio-Rad).

Removal of carbohydrate

Samples (15 μ g) of freeze-dried, purified SSAO were heated at 100 °C for 3 min in the presence of 0.1% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. The samples were diluted with 20 mM phosphate buffer/10 mM Na₂N₃/50 mM EDTA/0.5% (w/v) Triton X-100, pH 7.2, and heated at 100 °C for 3 min. After cooling, 0.5 units of *N*-glycosidase F or 0.5 units of *N*-glycosidase F, 0.5 units of *O*-glycosidase and 0.1 units of neuraminidase were added and incubated for 20 h at 37 °C. SDS sample buffer containing 5% (v/v) 2-mercaptoethanol was then added to each tube and the samples were heated at 100 °C for 3 min before analysis by SDS/PAGE.

Cu determination

Samples containing 23 μ g of pure bovine lung SSAO (57.5 pmol) or 20 μ g of bovine serum amine oxidase (obtained from Sigma and further purified by chromatography in a Superdex 200 HR FPLC system) were assayed. Both protein solutions were dialyzed extensively against 20 mM potassium phosphate buffer, pH 7.2, containing 0.1% (w/v) Triton X-100. A sample of the final dialysis buffer (0.5 ml) was saved as a blank for Cu analysis. All buffer solutions were rendered metal free by passage through a column of the metal-chelating resin Chelex 100. Cu content was determined using an atomic absorption spectrophotometer (Varian SpectraAA.30) equipped with a granite furnace and ZEEMAN corrector. The spectral line chosen was 324.8 nm and the standard addition method was used.

Half-Cu-depleted bovine plasma and membrane-bound SSAOs were prepared as described by Morpurgo et al. [30].

RESULTS

Solubilization of SSAO from bovine lung microsomes

After treatment of the microsomal membranes with either different concentrations of KCl or NaCl (0.1–3 M) or a hypotonic buffer, followed by centrifugation at 105 000 *g* for 1 h, over 90% of the SSAO activity was found in the resulting pellet, which suggests that the enzyme is not a peripheral membrane protein. Therefore attempts were made to determine whether the enzyme was anchored to the microsomal membrane by a membrane-spanning domain, or by a phosphatidylinositol–glycan type linkage. Treatment of membranes with phosphatidylinositol-specific phospholipase C did not solubilize the SSAO activity, even when ten times the recommended lipase units were used [31,32]. Treatment with phospholipase A₂, C or D also failed to release the membrane-bound enzyme. Furthermore, incubation of the solubilized enzyme with different concentrations of these phospholipases did not affect the activity, suggesting a lack of phospholipid dependence. Thus it was concluded that SSAO is an integral membrane protein, as has been suggested for the enzyme from rat brown adipose tissue [7].

Table 1 Purification of SSAO from bovine lung microsomes

The results shown are representative values from one of five separate preparations.

| Purification step | Total protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min per mg of protein) | Relative specific activity | Yield (%) |
|--------------------------------|--------------------|---------------------------|--|----------------------------|-----------|
| Microsomes | 1500 | 1650 | 1.1 | 1 | 100 |
| Triton solubilization | 600 | 1500 | 2.5 | 2.3 | 90 |
| Cibacron Blue 3GA-agarose | 62 | 1080 | 17.5 | 16 | 72 |
| Hydroxylapatite | 15 | 750 | 48 | 44 | 50 |
| <i>Lens culinaris</i> -agarose | 3.5 | 390 | 112 | 102 | 26 |
| Resource Q-FPLC | 1.5 | 330 | 210 | 190 | 22 |
| Superdex 200-FPLC | 0.9 | 225 | 250 | 230 | 15 |

Detergents were used at various concentrations in an attempt to solubilize the enzyme. Optimal concentrations of 1%, 1% and 0.6% were determined for β -octylglycoside, sodium cholate and Triton X-100 respectively. When sodium cholate was used, the best results were obtained when 150 mM NaCl was also included. The yield of the extracted enzyme activity exceeded 90% in all three cases.

The presence of detergent in all buffers was found to be necessary for maintaining SSAO activity. Sodium cholate was not compatible with ion-exchange chromatography and octyl glycoside cannot be used with affinity lectin chromatography, therefore, 0.6% (w/v) Triton X-100 (Triton:protein, 1.2% (w/w)) was chosen for further purification of SSAO. The K_m values for SSAO for benzylamine (K_m , 40 μ M) and 2-phenylethylamine (K_m , 312 μ M) were found to be unchanged by solubilization with 0.6% (w/v) Triton X-100. The soluble enzyme also showed similar sensitivities to the hydrazine-type inhibitors semicarbazide, phenelzine and phenylhydrazine.

The solubilized SSAO had a high stability and could be stored at 4 °C for at least one week without significant loss of activity. Therefore, since the purified enzyme was insensitive to proteolytic digestion, no protease or peptidase inhibitors were included during the purification procedure.

Purification of SSAO

The results of the purification procedure are summarized in Table 1. SSAO was purified 230-fold from the microsomal membranes (about 1000-fold with respect to the tissue homogenate) with an overall yield of 15%. The specific activity of the final enzyme preparation was 250 nmol min⁻¹/mg⁻¹ of protein under standard assay conditions. This preparation was stable for at least 12 months when stored at –80 °C.

During chromatography on hydroxylapatite about 70% of SSAO activity did not bind to the resin (Figure 1, upper panel). This unretarded material had a specific activity of 48 nmol min⁻¹·mg⁻¹ and a 2.75-fold purification was achieved. However, 30% of the enzyme was tightly bound to the hydroxylapatite and high molarity (0.4 M) phosphate buffer was necessary to elute it. The unretarded material again produced two peaks when re-chromatographed on the same column. The enzyme from each peak gave similar K_m values for benzylamine and 2-phenylethylamine oxidation. Furthermore, each had the same sensitivity to inhibition by the carbonyl-group inhibitors semicarbazide, hydralazine, phenylhydrazine and phenelzine, and similar thermal stability. This heterogeneous chromatographic behaviour is similar

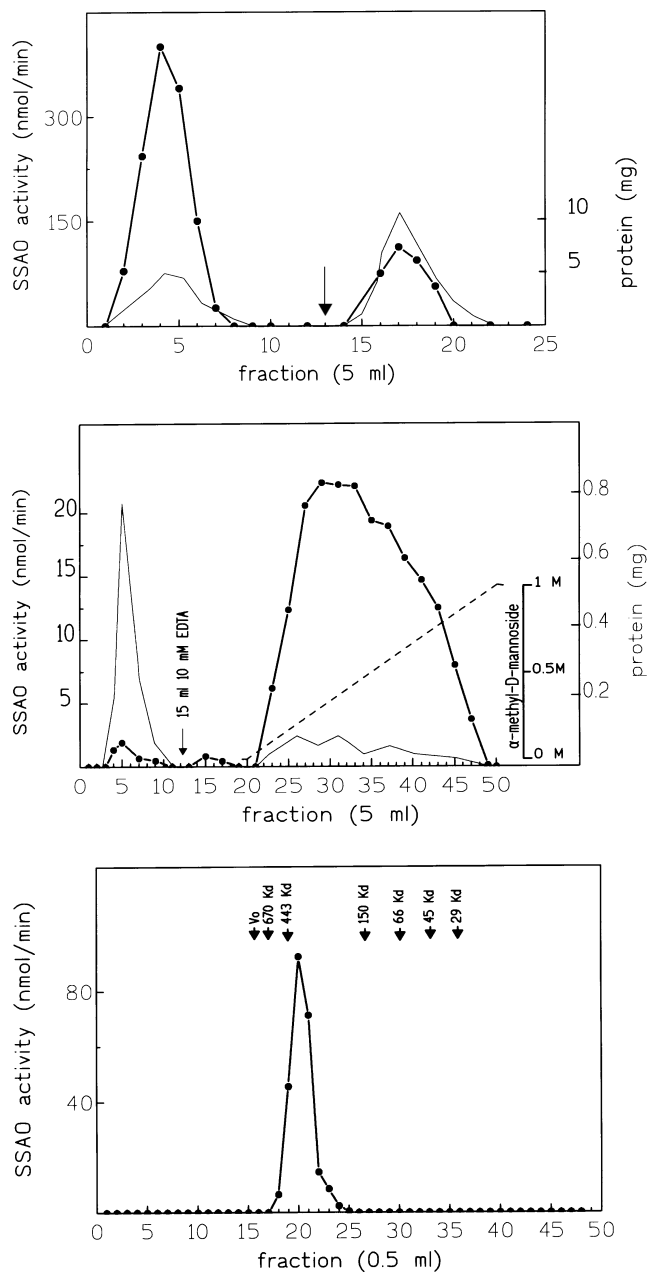


Figure 1 Elution profiles of SSAO on (upper panel) hydroxylapatite, (middle panel) *Lens culinaris*-agarose and (bottom panel) Superdex 200 HR-FPLC

Fractions were assayed for protein content (—) or for SSAO activity with 20 μ M benzylamine as substrate (●). (Upper panel) The molarity of the potassium phosphate buffer was increased to 0.4 M at the point indicated by an arrow. (Middle panel) Changes in the concentration of α -methyl-D-mannopyranoside are indicated by the broken line. (Bottom panel) Arrows indicate the positions of the void volume (V_0) and molecular mass markers.

to that described by Falk et al. [26] for pig plasma SSAO, when it was concluded that differences in the carbohydrate content were responsible. The unretarded material was further purified.

Preliminary experiments showed that the Triton X-100-solubilized microsomal SSAO bound to several immobilized lectin resins, suggesting a high degree of heterogeneity in the carbohydrate content. About 95% of enzyme bound to either *Lens culinaris*-agarose or Concanavalin A-Sepharose 4B resin and was specifically eluted in a broad peak using a continuous

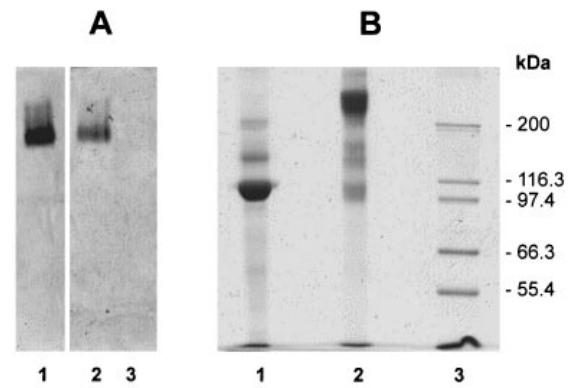


Figure 2 Electrophoresis of purified SSAO

(A) Non-denaturing PAGE (5% separating gel) of 10 μ g of purified SSAO. The gel was stained for protein with silver (lane 1), for enzyme activity (lane 2) or for enzyme activity including the inhibitor phenylhydrazine in the reaction mixture (lane 3) as described in the Experimental section. (B) SDS/PAGE (7.5% gel) of purified SSAO; lane 1, 5 μ g of purified SSAO under reducing conditions; lane 2, 5 μ g of purified SSAO under non-reducing conditions; lane 3, molecular mass markers. Protein was visualized by silver staining.

gradient of 0–1 M α -methyl-D-mannopyranoside. However, chromatography on Con A gave a poor yield (35%) when compared with that when *Lens culinaris* resin was used. Such a difference in the yield for this type of chromatography is similar to that described for the brown adipose tissue SSAO [7] and could be due to the fact that, although both lectins bind the same sugar residues, mannose and methyl-mannose derivatives, Con A has an affinity that is about 50 times higher [33]. On *Lens culinaris*-agarose chromatography, SSAO activity eluted as a broad peak with the mannose gradient (Figure 1, middle panel), indicating a high degree of heterogeneity in the carbohydrate content of the enzyme. Furthermore, when solubilized microsomal SSAO was chromatographed on a galactosamine-specific wheat germ lectin-agarose resin, two separate peaks of enzyme activity were resolved, using either 0.1 or 0.5 M galactosamine (results not shown).

Characterization of pure SSAO

PAGE under non-denaturing conditions revealed a single band after silver-staining coincident with the position of the band seen after staining for enzyme activity (Figure 2A). This band of enzyme activity did not appear when the SSAO inhibitor phenylhydrazine was included in the activity-stain mixture. A single symmetrical protein peak was obtained on FPLC-gel filtration, which corresponded to a molecular mass of 400 kDa (Figure 1, lower panel). However, SDS/PAGE analysis of the purified enzyme under reducing conditions (Figure 2B) revealed three bands with apparent molecular masses of 100, 130 and 170 kDa. These bands did not stain equally; the 100 kDa band accounted for 87% of the total stain (using either silver or Coomassie Blue), whereas the 130 and 170 kDa bands accounted for about 8% and 5% of the total staining intensity respectively. Furthermore SDS/PAGE analysis under non-reducing conditions (Figure 2B, lane 2) showed a major band (75% of total stain) with an apparent molecular mass of 250 kDa and 4 minor bands that accounted for 6% total stain each. Treatment of the enzyme with chaotropic agents (6 M BrLi, 8 M urea), 2% (w/v) SDS, 2.5% (v/v) 2-mercaptoethanol or 2% (w/v) SDS plus 2.5% (v/v) 2-mercaptoethanol and heating at 50 $^{\circ}$ C for 1 h, affected neither the apparent molecular mass of the enzyme nor

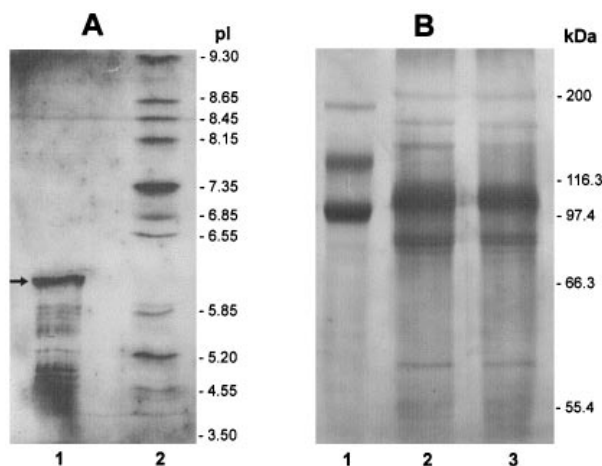


Figure 3 Microheterogeneity analysis of purified SSAO

(A) Native isoelectric focusing/PAGE electrophoresis: lane 1, 15 μ g of purified SSAO; lane 2, isoelectric point markers. Protein was visualized by staining with Coomassie Blue R250 as described in the Experimental section. The arrow indicates the sample application point. (B) SDS/PAGE (7.5% gel) of purified SSAO treated with glycosidases. Lane 1, 5 μ g of SSAO; lane 2, 15 μ g of SSAO treated with *N*-glycosidase F; lane 3, 15 μ g of SSAO treated with *O*-glycosidase F, *O*-glycosidase and neuraminidase. Protein was visualized by silver staining.

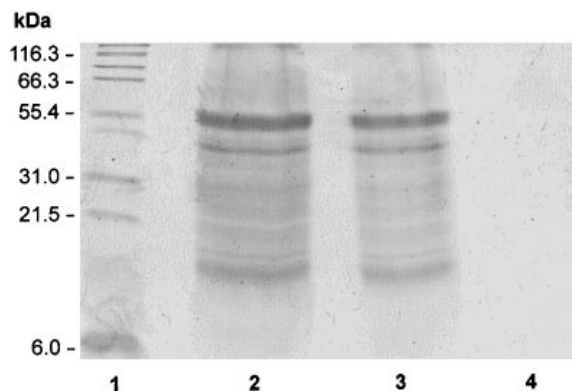


Figure 4 Electrophoretic pattern of CNBr-cleavage fragments of 100, 130 and 170 kDa subunits of purified SSAO

CNBr-cleavage fragments were prepared from bands eluted from a CD-Immobilion membrane and analysed on SDS/PAGE (15% gel), as described in the Experimental section. Lane 1, molecular mass markers; lane 2, cleavage of 100 kDa subunit; lane 3, cleavage of 170 kDa subunit; lane 4, cleavage of 130 kDa subunit. Protein was stained with Coomassie Blue R250.

the band pattern seen on SDS/PAGE. Sequencing of the N-terminal residues was unsuccessful, suggesting that they were blocked.

In attempts to remove possible contaminating protein, the purified enzyme was subjected to reverse-phase HPLC on a C4 or C18 column. However, the protein bound tightly to both of these resins and could not be eluted with acetonitrile, even increased to 100%, whereas Triton X-100, applied with the enzyme, eluted at an acetonitrile concentration of approx. 60%. The apparently irreversible binding of some membrane proteins to these hydrophobic chromatography materials have been reported by others [34].

Protein staining after isoelectric focusing of the purified enzyme gave several bands ranging from pH 4–6 (Figure 3A). All bands

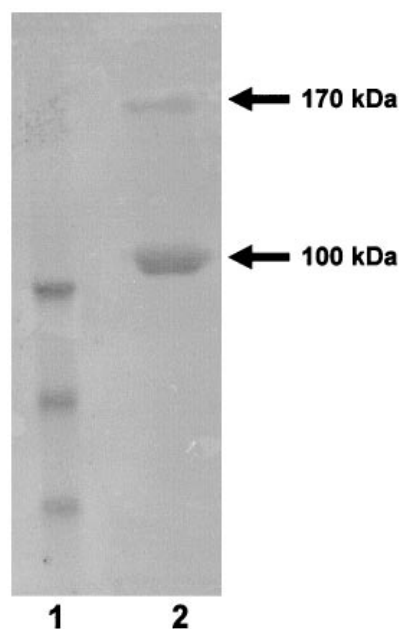


Figure 5 Redox-cycling staining of SSAO

Purified SSAO was resolved on SDS/PAGE (7.5 gel) under reducing conditions, electroblotted on to a nitrocellulose membrane and stained for quinone according to the method of Paz et al. [27]. Lane 1, pre-stained molecular-mass markers; lane 2, 20 μ g of purified SSAO.

stained for enzyme activity. A similar heterogeneity has also been described for the pig plasma amine oxidase [26] and for human placental diamine oxidase [35], which might be explained by the glycoprotein nature of these enzymes, since a few differences in the content of charged carbohydrate components of the enzyme would result in heterogeneity of the isoelectric points. Treatment of previously denatured enzyme with *N*-glycosidase F and/or *O*-glycosidase resulted in changes in the molecular masses of all three subunits and resulted in a greater heterogeneity upon SDS/PAGE analysis (Figure 3B).

When the three protein-staining bands were isolated and subjected to CNBr digestion, the 100 and 170 kDa bands yielded identical peptide patterns after chemical cleavage (Figure 4), suggesting the larger band was an incompletely dissociated dimer of the 100 kDa subunit. No material was detected after similar treatment of the minor 130 kDa band. Attempts to examine the peptides produced by proteolytic cleavage were unsuccessful, since no significant digestion was observed after incubation with either trypsin or chymotrypsin solutions at protein:protease ratios up to 5:1. Neither was the activity of the solubilized enzyme affected by such treatments.

Cofactor studies

'Redox-cycling' staining is a specific method for detecting quinoproteins and it has been used previously for the identification of the quinone cofactor of several amine oxidases [12,27,36]. This method is based on the ability of quinones to catalyse the reduction of Nitro Blue Tetrazolium at alkaline pH in the presence of excess glycine as reductant. Figure 5 shows the quinone staining of purified SSAO which had been electroblotted on to a nitrocellulose membrane following SDS/PAGE. The 100 and 170 kDa bands stained for quinone but the 130 kDa band did not. When the enzyme was treated with phenylhydrazine, a

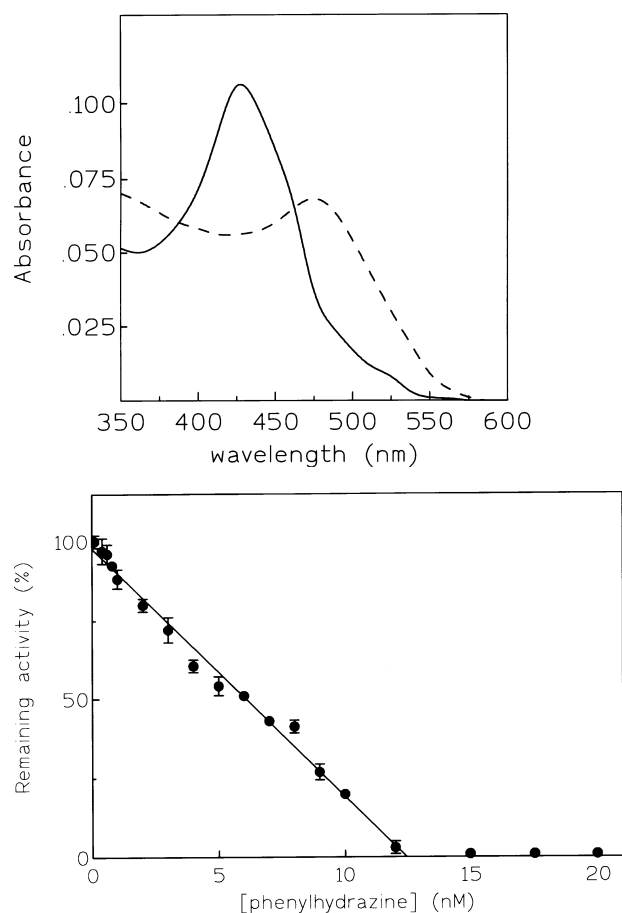


Figure 6 Analysis of the properties of the cofactor of purified SSAO

(Upper panel) Absorbance spectra of the phenylhydrazine derivative of purified SSAO. Purified SSAO (1 mg) was dissolved in 2 ml of potassium phosphate buffer, pH 7.2. Ten molar equivalents of phenylhydrazine hydrochloride were added to the enzyme and the mixture was incubated at 37 °C for 30 min. Unreacted phenylhydrazine was removed by dialysis against the same buffer. Visible absorption spectra of the adduct before (solid line) and after (broken line) the addition of 2 M KOH. (Lower panel) Titration of purified SSAO with phenylhydrazine. The enzyme (15 nM) was dissolved in 50 mM potassium phosphate buffer, pH 7.2 and titrated with freshly prepared solutions of phenylhydrazine. After incubation for 15 min at 37 °C, the remaining activity was determined with 20 μ M benzylamine as substrate. Each value is the mean \pm S.D. from three separate enzyme preparations.

maximum of 425 nm in the visible spectrum of the derivatized SSAO was observed at neutral pH and this shifted to 463 nm when 1 M KOH was added (Figure 6, upper panel). These spectral changes are somewhat lower than has frequently been observed with protein- or peptide-bound phenylhydrazones (443–447 at neutral pH and about 482 in alkali [37]). These differences could result from the presence of detergent that was necessary to maintain the solubility and activity of this enzyme. An alternative procedure, involving topa quinone detection by reaction with *p*-nitrophenylhydrazine followed by absorbance spectra determinations at neutral pH and in the presence of 2 M KOH [2], could not be used in the present case, since the presence of Triton X-100 in the enzyme preparation resulted in turbidity. When the enzyme activity was titrated with phenylhydrazine (Figure 6, lower panel) a stoichiometry of 0.83 mol of phenylhydrazine/mol of native enzyme (400 kDa) was obtained.

Cu content was determined by atomic absorption spectrophotometry using pure SSAO with bovine plasma amine oxidase,

Table 2 Cu content and enzyme activities of native, half-copper depleted and reconstituted preparations of SSAO from bovine plasma (BSAO) and lung (LSSAO)

The Cu content was determined assuming molecular masses of 400 and 170 kDa for the ox lung and plasma enzymes respectively. Experimental details are given in the text.

| Sample | Cu content (mol/mol enzyme) | Activity (%) |
|--------------------------------|-----------------------------|--------------|
| Native BSAO | 1.75 | 100 |
| DDC + dithionite-treated BSAO | 0.95 | 5 |
| Reconstituted BSAO | 1.88 | 85 |
| Native LSSAO | 1.84 | 100 |
| DDC + dithionite-treated LSSAO | 1.20 | 10 |
| Reconstituted LSSAO | 1.91 | 73 |

which contains 2 mol of Cu/mol of native enzyme [37] as a control (Table 2). Pure bovine lung SSAO was found to contain 1.84 ± 0.06 mol of Cu/mol of native enzyme (400 kDa), close to the value obtained with the plasma enzyme (1.75 ± 0.05 mol of Cu/mol of enzyme). This is the first report on the presence of Cu in the tissue-bound SSAO.

Dialysis of pure bovine plasma SSAO against 1 mM *N,N*-diethyldithiocarbamate (DDC) under anaerobic reducing conditions, as described by Morpurgo et al. [30], resulted in the removal of about half the Cu content of the protein and an 80 % decrease in enzyme activity. This treatment had no significant effect on either Cu content or enzyme activity of the bovine lung enzyme. However, it was found that increasing the DDC concentration to 2.5 mM, under the same conditions, was effective in depleting the Cu content of the preparation to about 50 % of that originally present and in almost completely abolishing the enzyme activity (Table 2). There was substantial recovery of the activities of the Cu-depleted preparations of both the bovine and plasma SSAO following dialysis against CuSO_4 . Treatment with 1 mM KCN or 1 mM DDC or dialysing against the metal-chelating resin, Chelex 100, which have been shown to inhibit the activity of plasma SSAO, were without effect on the activity of the purified bovine lung enzyme. The IC_{50} of KCN for the bovine lung enzyme was found to be 5 ± 1 mM, whereas the value determined in parallel experiments for the bovine plasma SSAO was 50 ± 15 μ M.

Immunological studies

The native SSAO purified from bovine lung was used to immunize rabbits and produced a high-titre polyclonal antibody that could detect less than 0.1 ng of antigen on ELISA. Using 1 ng of antigen, the antiserum was positive at a dilution greater than 1:32000. Western-blot analysis revealed that the 100 and 170 kDa bands were recognized by this antibody, with the 100 kDa subunit staining most intensely (Figure 7A). Human lung and bovine plasma SSAO were also recognized by these antibodies (Figures 7A and 7B respectively). The antiserum raised to pig plasma amine oxidase cross-reacted poorly with bovine lung SSAO (a 1:10 dilution of antiserum had to be used) and recognized the 100 and 170 kDa bands (Figure 7C).

Kinetic studies

Table 3 shows the kinetic constants (K_m , k_{cat} , k_{cat}/K_m) for the oxidation of benzylamine, methylamine, 2-phenylethylamine and histamine by purified SSAO. The enzyme had the lowest K_m value towards benzylamine. However, methylamine and ben-

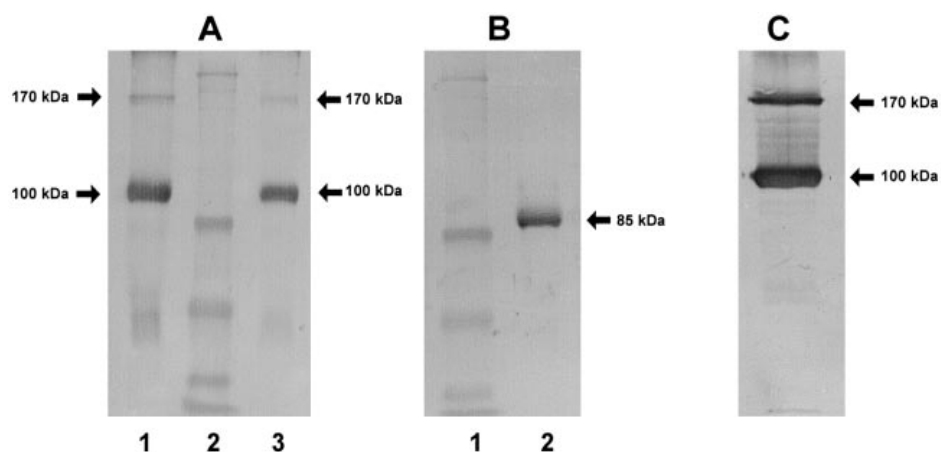


Figure 7 Immunoblot analysis of SSAO

Immunoblot analysis was performed using rabbit anti-bovine lung SSAO (**A** and **B**) or rabbit anti-pig plasma SSAO antibodies (**C**) as described in the Experimental section. (**A**) Lane 1, 100 μg of bovine lung homogenate; lane 2, pre-stained molecular-mass markers (Novex); lane 3, 100 μg of human lung homogenate. (**B**) Lane 1, pre-stained molecular-mass markers (Novex); 5 μg of partially-pure bovine plasma SSAO. (**C**) Purified bovine lung SSAO (10 μg). Proteins were electroblotted after SDS/PAGE 7.5% gel and stained as described in the Experimental section.

Table 3 Kinetic constants of purified bovine lung SSAO

Experimental details are given in the text. Each value is the mean \pm S.D. from determination from three separate enzyme preparations. *Data from Hartman and Klinman [54].

| Substrate | K_m (μM) | k_{cat} (min^{-1}) | k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$) |
|----------------------------|-------------------------|--|---|
| Benzylamine | 50 ± 2 | 170 ± 22 | 3.40 |
| Methylamine | 210 ± 10 | 580 ± 40 | 2.76 |
| 2-Phenylethylamine | 1187 ± 3 | 62 ± 7 | 0.33 |
| Histamine | 1110 ± 35 | 110 ± 5 | 0.10 |
| Benzylamine (plasma SSAO)* | 730 | 69 | 0.095 |

Table 4 Inhibition of purified SSAO by amiloride and some hydrazine derivatives

The IC_{50} is the inhibitor concentration necessary to give 50% inhibition under the assay conditions used. The concentration of crude enzyme and purified enzyme were 1.5 and 0.01 mg/ml respectively. Each value is the mean \pm S.D. from three separate enzyme preparations.

| Inhibitor | Preincubation (min) | IC_{50} crude enzyme (μM) | IC_{50} pure enzyme (μM) |
|-----------------|---------------------|---|--|
| Amiloride | 0 | > 50 000 | 750 ± 100 |
| Semicarbazide | 30 | 10 ± 3 | 10 ± 2 |
| Semicarbazide | 0 | 100 ± 12 | 80 ± 15 |
| Phenelzine | 0 | 0.020 ± 0.004 | 0.006 ± 0.002 |
| Phenylhydrazine | 0 | 0.025 ± 0.009 | 0.0005 ± 0.0001 |

zylamine gave similar specificity constants (k_{cat}/K_m), and the maximum velocity was highest with the methylamine as substrate. Histamine was the poorest of these substrates. In the case of benzylamine, inhibition by high substrate concentration was observed and a substrate-inhibition constant (K_{si}) of $1850 \pm 80 \mu\text{M}$ was determined. The enzyme did not oxidize dopamine, tyramine, tryptamine, kynuramine or 5-hydroxytryptamine to any detectable extent.

Table 4 summarizes the inhibition of both crude and pure

preparations of the bovine lung enzyme by some SSAO inhibitors. The carbonyl reagents, semicarbazide, a time-dependent and irreversible inhibitor, and phenelzine, a reversible inhibitor, showed similar IC_{50} values toward both enzyme preparations. However, the purified enzyme was about 50 times more sensitive to inhibition by phenylhydrazine and amiloride was more than 100 times more potent with that enzyme preparation. The specific MOA inhibitors clorgyline and deprenyl [22] did not affect the activity of the bovine lung enzyme.

DISCUSSION

Previous work from this laboratory has shown SSAO from bovine lung to have a relatively high activity and to be located in the microsomal fraction [13]. The microsomal enzyme appears to be an integral membrane protein, since enzyme activity was released into supernatant by the use of detergents (Triton X-100, sodium cholate or β -octyl glycoside) whereas mild treatments, such as increasing the ionic strength of the medium, were without effect.

In this study an apparently pure preparation of SSAO (shown by non-denaturing electrophoresis, Figure 2A) has been obtained with an average of yield of 0.9 mg of enzyme protein and with a specific activity of $0.25 \mu\text{mol min}^{-1} \cdot \text{mg}^{-1}$ of protein (unit/mg). At saturating (V_{max}) concentrations of benzylamine the specific activity was 0.425 unit/mg, a value comparable to those of 0.22 and 0.41 units/mg of protein reported for pure preparations of SSAO from pig and bovine plasma respectively [38,39]. This study is the first reported procedure for the extensive purification of membrane-bound SSAO and should facilitate comparison with the soluble enzymes from plasma and tissues.

The native enzyme exhibited an apparent molecular mass of 400 kDa by gel filtration (Figure 1, lower panel), whereas SDS/PAGE yielded a major band of 100 kDa and two minor ones of 130 and 170 kDa (Figure 2B). Chemical cleavage of the 170 and 100 kDa bands with CNBr yielded identical peptides (Figure 4). This result, together with the data showing that both the 100 and 170 kDa bands contain a quinone cofactor (Figure 5) and bind the antibodies raised to purified bovine lung and pig plasma SSAO preparations (Figure 7A), would suggest that the larger band is an incompletely resolved dimer of the 100 kDa

subunit. The 130 kDa band, which accounted for only 8% of the total stain after SDS/PAGE, did not contain a quinone, cross-react with the SSAO antibodies or yield detectable peptides following CNBr treatment. It may represent a minor impurity that tightly associates with the SSAO, although it is also possible that it may constitute an electrophoretic artefact. Anomalous banding patterns on SDS/PAGE that are unrelated to sample purity have been discussed by others [40].

The results presented here are consistent with SSAO being a tetramer composed of 100 kDa subunits. This value is close to the 97 kDa that has been reported recently for the cloned SSAO associated with the GLUT 4 glucose transporter from adipocyte plasma membrane [18]. Several authors have suggested that the tissue-bound enzyme could be the precursor form of the plasma amine oxidase [36,41,42], but all purified soluble SSAO proteins have been shown to be comprised of two identical subunits with an molecular mass for the native enzyme of 170–190 kDa (see [37] and [43] for reviews). In contrast to the results reported here, gel filtration and exposure of membrane-bound SSAO from rat brown adipose tissue to irradiation inactivation indicated an estimated molecular mass of 183 kDa [7]. The observation of an apparent value of 250 kDa after PAGE under non-reducing conditions might indicate that the tetrameric species is composed of two more tightly-associated dimers. Since the behaviour of the enzyme indicated it to be a glycoprotein, it is important to bear in mind that heavily glycosylated proteins may behave anomalously on gel-filtration and on electrophoresis in the presence of SDS [44]. This explanation has also been advanced to account for the apparent heterogeneity of active species of soluble SSAO from pig plasma [26] and diamine oxidase from human placenta [35], and may also account for the multiplicity of bands seen on isoelectric focusing, although differential binding of ampholine to the enzyme might also contribute to this [45].

Plasma amine oxidases contain a covalently bound organic cofactor, the structure of which has been identified recently as the quinone of (2,4,5-trihydroxyphenyl)-L-alanine (topa quinone) [1]. Topa quinone is integrated into the polypeptide chain and is encoded by a tyrosine codon [46,47], with the post-translational generation of cofactor from the precursor tyrosine residue occurring by an, as yet, unknown mechanism [41]. The results presented here indicate that bovine lung SSAO has a quinone-like cofactor. The visible spectrum of this enzyme after reaction with phenylhydrazine (Figure 6, upper panel), was similar to, but not identical with, that reported for the plasma enzyme [2]. Although this might result from the presence of Triton X-100, which was necessary to maintain the solubility and activity of the lung enzyme, or from differences in the structure of these two enzymes, it would be necessary to obtain amino-acid sequence data to establish the presence of topa quinone definitively.

Titration of the enzyme with phenylhydrazine indicated complete inhibition at a ratio of 0.83 mol of phenylhydrazine/mol of native enzyme (400 kDa) (Figure 6, lower panel). Earlier studies with purified serum SSAO preparations, which are known to contain two active sites per dimer [48], also gave ratios of approx. one mol of inhibitor/mol of enzyme at complete inhibition, and were interpreted in terms of the dimeric enzyme showing half-of-the-site reactivity [49–51]. However, Janes and Klinman [39], who found that two mol phenylhydrazine/mol of enzyme were required to inhibit purified bovine plasma SSAO, suggested that the half-of-the-site reactivity observed by others might have been due to the presence of inactive subunits in those preparations. This discrepancy may result from differences in the reactivities of the two topa quinone-containing subunits in the dimeric bovine plasma SSAO, only one of which has been reported to react rapidly with hydrazine inhibitors in a process that results in

complete loss of activity [52,53]. Although the possibility that purified lung enzyme contained inactivated subunits cannot be excluded, this preparation had a specific activity that was comparable with that reported for the bovine plasma preparation of Janes and Klinman [39].

KCN has been used to differentiate tissue-bound from soluble SSAO enzymes, since 1 mM KCN inhibits the plasma enzyme irreversibly by interaction with the tightly-bound Cu that is necessary for enzymic activity [54]. In contrast, CN⁻ has been reported not to affect the activity of crude preparations of the membrane-bound enzyme [55]. Because of this, and because the Cu chelators diethyldithiocarbamate and penicillamine were found to have no effect on the activity of membrane-bound SSAO, some authors have suggested that this form of SSAO may not contain Cu [7,42]. The atomic absorption spectrophotometry studies reported here showed clearly that bovine lung SSAO contained 2 mol of Cu/mol of enzyme. These results are consistent with the involvement of Cu ions in the catalytic mechanism of the tissue-bound enzyme, perhaps playing a similar role to those in plasma SSAO. Removal of half of the Cu content of the purified tissue-bound SSAO, using DDC under anaerobic and reducing conditions, caused enzyme inactivation that was recovered by dialysis against CuSO₄, indicating that the two Cu ions were not equivalent. Similar behaviour has been reported for the purified bovine plasma SSAO [30]. However, it was necessary to use DDC concentrations that were 2.5 times higher than those reported to be effective with the plasma enzyme [30] for depleting the Cu content of the purified bovine lung SSAO. Furthermore, the relative insensitivity of the lung enzyme to inhibition by KCN (IC₅₀ value some 100 times higher than that for the plasma enzyme) and Cu-chelating agents might suggest that the metal is effectively shielded by the folded structure of the enzyme.

Mammalian plasma and tissue-bound SSAOs can deaminate both aromatic and aliphatic primary amines. Benzylamine is often regarded as being the best substrate and the plasma SSAO is sometimes referred to as benzylamine oxidase [8,20,38]. The purified enzyme from bovine lung had a particularly high specificity constant (k_{cat}/K_m ; based on a molecular mass of 400 kDa) for this substrate, which was some 36 times higher than that reported for pure bovine plasma SSAO [54]. However, methylamine was found to have a specificity constant for the bovine lung SSAO which was comparable with that of benzylamine and a catalytic activity which was considerable higher (Table 4). This is consistent with the proposed involvement of the lung enzyme in the oxidation of inhaled volatile amines [15]. The bovine lung enzyme had a much narrower substrate specificity than that reported for the soluble SSAO from several sources [56,57] and had no significant activity towards dopamine, 5-hydroxytryptamine, tryptamine or tyramine.

IC₅₀ values depend on the nature of the inhibitory process and, in the case of irreversible inhibitors, on the molar concentration of enzyme. However, provided that the type of inhibition is known, they do provide a useful guide to relative potencies under any set of defined conditions, such as those used in the present studies [58]. Although the sensitivity of the bovine lung enzyme to inhibition by semicarbazide was not significantly affected by the extraction and purification procedure, the purified preparation had a markedly higher sensitivity to inhibition by the hydrazine derivatives phenelzine and phenylhydrazine, as well as to inhibition by amiloride. Although the sensitivity towards the irreversible inhibitor phenylhydrazine might reflect other components of the crude preparation reacting with this compound, the nature of the factors resulting in the increased inhibition by phenelzine and amiloride requires more detailed kinetic studies,

since these compounds interact with the enzyme in different ways. Phenelzine is a reversible inhibitor which exhibits complex kinetic behaviour [59], whereas amiloride is an apparently simple reversible inhibitor. Whether these changes simply reflect removal of the enzyme from its membrane environment, affecting inhibitor partitioning and consequent local concentration effects, or result from modifications during the purification procedures used will require further studies.

Amiloride and some other imidazoline and guanidine compounds are inhibitors of Na⁺-transporting systems. They have proved useful for studies on the mechanism and physiology of Na transport and, clinically, as diuretic and hypertensive agents [60]. It now appears that both MAO and SSAO may function as tissue amiloride- [61] and imidazoline-binding proteins [62]. Several studies have described the inhibition of plasma SSAO and MAO enzymes by amiloride and related compounds [36]. In contrast to the potency of amiloride as a reversible inhibitor for plasma SSAO (K_i 196 μ M [36]) and human kidney diamine oxidase (IC_{50} 5 μ M [61]), amiloride was a poor inhibitor of the bovine lung enzyme (Table 4). This difference might prove useful for distinguishing between the soluble and tissue-bound enzymes in tissue preparations containing both.

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