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Solubilization and some properties of a semicarbazide-sensitive amine oxidase in brown adipose tissue of the rat

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A semicarbazide-sensitive clorgyline-resistant amine oxidase (SSAO) was solubilized from membrane fractions of rat brown adipose tissue by the non-ionic detergent, Triton X-100. Alteration of ionic strength or addition of chelating agents alone failed to release the enzyme from its membrane. Lipid-depletion led to loss of enzyme activity and alteration of substrate affinity. Over 80% of the activity of the solubilized enzyme was found in gel filtration fractions corresponding to an M_r of between 160000 and 180000. The glycoprotein nature of SSAO was established from affinity chromatography with either immobilized concanavalin A or *Lens culinaris* lectin. Elution of over 50% SSAO activity from the lentil lectin was achieved with 0.25M-α-methyl D-mannoside to give 80–90-fold purification of the enzyme. Irradiation inactivation gave a value for M_r of around 183000 for both soluble and membrane-bound SSAO. Substrate affinity and inhibitor sensitivity of the enzyme were unaltered by solubilization. The copper-chelating agent, diethyldithiocarbamate, did not affect the enzyme, shedding doubt on the suggestion that SSAO is a copper-requiring enzyme. The significance of these findings in relation to the nature of SSAO and to its disposition within the cell membrane is discussed.

An amine oxidase distinct from MAO (amine:oxygen oxidoreductase, EC 1.4.3.4) has been identified in homogenates of brown adipose tissue of the rat (Barrand & Callingham, 1982). This enzyme is resistant to inhibition by the irreversible MAO inhibitor, clorgyline; hence we have called it clorgyline-resistant amine oxidase. Unlike MAO it is inhibited by carbonyl reagents, such as semicarbazide, and may be referred to as semicarbazide-sensitive amine oxidase (SSAO). So far, benzylamine appears to be the substrate with the highest affinity for the enzyme.

Benzylamine is a good substrate for a variety of non-MAO amine oxidases, including those found in, for example, pig, sheep, ox and human plasma, pig kidney, human placenta, ox and pig aorta and dental pulp (Blaschko, 1974; Nakano *et al.*, 1974; Yasunobu *et al.*, 1976; Sourkes, 1980; Norqvist *et al.*, 1982). Most of the enzyme activities extracted from these tissues can metabolize diamines and polyamines. Some, such as the ox dental pulp enzyme (Nakano *et al.*, 1974), can also deaminate lysyl-vasopressin, a substrate of the connective tissue enzyme lysyl oxidase, which is specifically involved in the cross-linking of collagen and elastin (Buffoni, 1980), but which cannot itself metabolize benzylamine. Brown fat SSAO, by virtue of its substrate specificity, differs from this group of enzymes.

Benzylamine-metabolizing activities more closely resembling that found in brown fat have been detected in several tissues of the rat, for example, skull (Andree & Clarke, 1981), anococcygeus muscle (Callingham, 1982), heart (Clarke *et al.*, 1982) aorta (Coquil *et al.*, 1973; Urdin & Fuentes, 1983) and mesenteric blood vessels (Fuentes & Neff, 1977; Callingham *et al.*, 1983). In addition a widespread distribution of a similar type of activity has also been reported to occur in man (Lewinsohn *et al.*, 1978). However, it is unknown whether or not a common enzyme is responsible. In many of these tissues the activity has been associated with smooth muscle, but in brown fat, recent evidence would suggest that the enzyme resides on the cell membranes of the brown adipocytes (Barrand *et al.*, 1983*b*).

In order to describe the SSAO enzyme in brown adipose tissue of the rat more precisely and to discover how closely it resembles these other

Abbreviations used: SSAO, semicarbazide-sensitive clorgyline-resistant amine oxidase; MAO, monoamine oxidase.

enzyme activities, solubilization and purification of the enzyme has been undertaken and its properties investigated.

A preliminary report of this work has already been published (Barrand *et al.*, 1983*a*).

Experimental

Tissue membrane preparation

Male Sprague–Dawley rats, 200–400g in weight, were killed by stunning followed by decapitation, and interscapular brown adipose tissue removed. The tissue was homogenized (1:20, w/v) in 1mM-potassium phosphate buffer (prepared by adjusting 1mM-KH_2PO_4 to pH7.8 with solid KOH at 22°C) and centrifuged at 5°C for 10min in a MSE Chilspin at 600*g* ($r_{av.}$ 11.3cm) to remove unbroken cells and cellular debris.

The supernatant from this spin formed the 'original homogenate'. 'Tissue membranes' containing the membrane-bound SSAO were prepared from the original homogenate by centrifuging in a MSE Prepspin at 4°C for 1h in an 8 × 50ml rotor operated at 100000*g* ($r_{av.}$ 7.62cm) or a 10 × 10ml rotor operated at 100000*g* ($r_{av.}$ 5.61cm). The protein content of the resultant pellet samples, redispersed in phosphate buffer, was estimated by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V from Sigma) as standard and adjusted to a final concentration of 1mg/ml for use in solubilization studies.

Solubilization studies

Samples of the 'tissue membranes' were mixed at 4°C with either water, 1mM-EDTA, 25mM-NaCl, 154mM-NaCl or 0.01, 0.02, 0.04, 0.07 and 0.14% (w/v) Triton X-100 in 20mM-phosphate buffer at pH7.8 and left for 10min before centrifuging as described above at 100000*g* for 1h. The resultant 'high speed' pellets and supernatants were taken for protein and amine oxidase assay. Where necessary, supernatant samples were concentrated in dialysis tubing by using Aquacide I-A (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.).

Lipid-depletion

Samples of the 'tissue membranes' were mixed with butan-2-one ('methyl ethyl ketone') as described by Ekstedt & Oreland (1976) in a ratio of 1:8 (v/v) and stirred rapidly. The ketone extract was poured off, evaporated to dryness and taken up in 1mM-phosphate buffer, pH7.8. The lipid-depleted residue was washed three times by suspending in the same phosphate buffer and spinning down at 600*g* for 10min before assay. Lipid depletion by acetone/water (15:1, v/v) or by acetone/water with 12μl of aq. NH_3 (sp.gr. 0.880) added/100ml was undertaken in a similar manner but with a 'tissue membrane' to solvent ratio of 1:24 (v/v) as described by Tong *et al.* (1979).

Gel filtration

This was carried out on a column (1.6cm × 48cm) of either Sephadex G-200 (effective fractionation range 5000–600000; Pharmacia, Uppsala, Sweden) or Ultrogel AcA 34 (effective fractionation range 20000–350000; LKB, Bromma, Sweden) with a flow rate of approx. 12ml/h. Columns were equilibrated with 20mM-phosphate buffer at pH7.8 with or without 0.1% Triton X-100 and calibrated with standard proteins; catalase, aldolase, bovine serum albumin and ovalbumin, the M_r of these being taken as 210000, 158000, 67000 and 43000 respectively. Blue Dextran 200 and Phenol Red were used to determine the void and bed volumes. Aliquots (1ml) of the 'high speed' supernatant obtained after solubilization with 0.08% (w/v) Triton X-100 and concentration to around 1mg of protein/ml were loaded on to the columns and eluted with the equilibration buffer. Fractions (1ml) were collected and subjected to protein and enzyme assay.

Affinity chromatography

This was undertaken with either Glycosylex A, an agarose–concanavalin A containing 17mg of concanavalin A coupled to 1ml of resin (Miles Laboratories, Slough, Berks., U.K.), or lectin from *Lens culinaris* immobilized on beaded agarose containing 4.5mg of lectin per ml of gel (Sigma Chemical Co., Poole, Dorset, U.K.). Aliquots (300μl) of solubilized enzyme, obtained by pooling and concentrating SSAO-rich fractions from gel filtration to between 0.2 and 0.4mg of protein/ml, were loaded on to 0.8ml of immobilized lectin already equilibrated with 20mM-phosphate buffer, pH7.8, containing 0.1% Triton X-100, 0.1mM-$MgCl_2$, 0.1mM-$MnCl_2$, 0.1mM-$CaCl_2$ and 0.5M-NaCl. A 5ml portion of this buffer was used to wash the gel. Elution of material bound to the lectin was achieved initially with 5ml of 0.25M-α-methyl D-mannoside in 0.02M-potassium phosphate buffer containing 0.1% Triton and 10mM-EDTA. Later a continuous gradient from 0 to 0.5M-mannoside was used. Aliquots (0.5ml) of the eluate were collected at all stages.

Irradiation inactivation

Samples of the 'original homogenate' or of the 'tissue membranes' or of 'high speed' supernatants obtained after solubilization with 0.08% Triton X-100 were freeze-dried overnight in glass-stoppered Pyrex tubes or in polypropylene microcentrifuge tubes and were irradiated under vacuum in a cooled aluminium block with the 16MeV linear electron accelerator MEL type SL 75/20 at New

Addenbrooke's Hospital, Cambridge. Done in this way, any indirect effects of irradiation were minimized. Doses up to 12Mrad were given at a rate of 2Mrad/min. For doses over 5Mrad, samples were irradiated in stages and were cooled between exposures to prevent overheating. After irradiation, samples were reconstituted in standard portions of distilled water and enzyme activity was assayed. Activity between samples was compared per volume of sample rather than per mg of protein, since apparent decreases in total protein content with increasing doses of irradiation were found on assaying for protein by the Lowry method. As might be expected, irradiation also modifies amino acid groups responsible for the colour changes in the Lowry method. Correct estimation of M_r relies on an accurate knowledge of the dose of irradiation administered. For this reason, internal standards, i.e. enzymes of known molecular weight, were assayed alongside the enzyme under investigation. MAO and glucose-6-phosphatase were found to be suitable as standards, since both enzymes are present in brown adipose tissue homogenates and both have already been subjected to M_r analysis by irradiation inactivation (Parkinson, 1979; Collipp *et al.*, 1974). Samples in which glucose-6-phosphatase was used as an internal standard were prepared in 1mM-Tris/HCl buffer, pH7.8, and not phosphate buffer to avoid high phosphate backgrounds in the enzyme assay.

The percentage of enzyme activity remaining after irradiation (in the form of the natural logarithm) was plotted against dose given (in rads). Linear regression analysis was used to determine the slope (k) of the plot and molecular size was calculated from the relationship empirically derived by Kepner & Macey (1968):

$$M_r = k \times 6.4 \times 10^{11}$$

Enzyme and Triton assay

Amine oxidase activity was determined radiochemically (Callingham & Laverty, 1973) with 0.025mM-[^{14}C]benzylamine (sp. radioactivity 10μCi/μmol) as substrate for SSAO and 0.5mM-5-hydroxy[^{3}H]tryptamine (sp. radioactivity 2μCi/μmol) as substrate for MAO-A, since previous experiments (Barrand & Callingham, 1982) showed that in this tissue the contribution of MAO-B activity towards the deamination of these substrates was negligible. For K_m determination, concentrations of benzylamine in the range 0.002–0.025mM were used. Inhibition studies were carried out by preincubating the samples at 37°C for 30min with appropriate dilutions of the inhibitors before addition of the substrate. Glucose-6-phosphatase activity was assayed by the spectrophotometric method of Parvin & Smith (1969) and Triton X-100 concentrations by the spectrophotometric method described by Wright & Plummer (1973).

Materials

5-Hydroxy[G-^{3}H]tryptamine creatinine sulphate and [1-^{14}C]benzylamine hydrochloride were 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

After treatment of the 'tissue membranes' prepared from brown adipose tissue with either water, 1mM-EDTA, 25mM-NaCl or 154mM-NaCl followed by centrifugation at 100000g for 1h, most of the SSAO activity was found in the resultant 'high speed' pellets and only trace amounts in the 'high speed' supernatant fractions, i.e. 94.9$\pm$0.2% (mean$\pm$S.E.M.) in pellets after water, 96.4$\pm$1.0% (mean$\pm$S.E.M.) in pellets after NaCl treatment and 96.5$\pm$0.1% (mean$\pm$S.E.M.) in pellets after EDTA treatment. Mild treatment was thus unable to remove the enzyme from its membrane.

Lipid depletion of the membrane however was found to affect SSAO activity. Treatment with either butan-2-one, acetone or acetone and ammonia caused significant loss of enzyme activity from the membrane, i.e. between 75% and 85% loss. A small amount of enzyme activity was found in the ketone extract. The substrate affinity of the membrane-bound enzyme was decreased (Table 1). Lipid depletion did not however modify the

Table 1. *K_m values for the deamination of benzylamine by membrane-bound, lipid-depleted and Triton-solubilized SSAO from adipose tissue of the rat*

Each value is the mean ($\pm$S.E.M.) obtained from a number (shown in parentheses) of separate preparations. Individual K_m values were derived by linear regression by the method of Wilkinson (1961). Five different substrate concentrations from 10^{-6}M to 2×10^{-5}M were used. Quadruplicate determinations were made of each point.

Source of SSAO	K_m (μM)
'Tissue membranes'	2.38$\pm$0.23 (6)
Lipid-depleted membranes	
(*a*) After butan-2-one treatment	6.75$\pm$1.05 (3)
(*b*) After acetone treatment	4.91$\pm$0.34 (3)
(*c*) After acetone/ammonia treatment	7.09$\pm$1.00 (3)
Supernatant after solubilization with 0.08% Triton X-100	2.45$\pm$0.12 (6)

sensitivity of the enzyme to inhibition by cuprizone (IC_{50} approx. 10^{-5}M). Nor was the lack of inhibition produced by 10^{-3}M-KCN and the small amount of inhibition (around 30%) produced by 10^{-3}M-β-aminopropionitrile on SSAO activity altered by lipid depletion.

Preliminary experiments on homogenates of brown adipose tissue revealed that SSAO activity was not inhibited by concentrations of Triton X-100 as high as 1% (w/v). SSAO activity was measured in 'high speed' pellets and in 'high speed' supernatant fractions after treatment with various concentrations of Triton followed by centrifugation at 100000*g* for 1h. SSAO remained in the pellet at low Triton concentration but at a Triton/protein ratio (w/w) of around 0.4 SSAO was released into the 'high speed' supernatant (Fig. 1). By contrast, MAO could not be detected in the supernatant fractions until a Triton/protein ratio of around 2 was used. At that Triton concentration there was a significant loss of total enzyme activity, i.e. activity in both pellet and supernatant together. The supernatant fractions obtained after treatment of membrane pellets at 1mg of protein/ml with 0.08% (w/v) Triton were concentrated with Aquacide to around 1–2mg of protein/ml and 1ml aliquots of this were subjected to gel filtration. At least 80% of the total SSAO activity applied could be recovered from the columns in the fractions corresponding to an M_r of between 160000 and 180000 (Fig. 2). When 0.02M-Tris buffer was used in place of the phosphate buffer for elution, the SSAO activity was found in the void volume. Removal of at least 80% of the Triton from the soluble enzyme preparation either by overnight dialysis against two changes of 200vol. of buffer alone or by $(NH_4)_2SO_4$ precipitation to 60% saturation followed by desalting through a Sephadex G-25 column also altered the filtration characteristics of the enzyme. On elution from a column of Ultrogel AcA 34, enzyme activity was no longer retained within the gel but appeared in the void volume. For these experiments, Sephadex G-200 could not be used because of the presence in the sample of something, presumably an enzyme, inactive in 0.1% (w/v) Triton but active in the presence of concentrations of Triton below 0.02% (w/v), which was capable of destroying some of the Sephadex linkages and which produced total destruction of the gel column. After testing the dissolved Sephadex gel for hexoses by the phenol/H_2SO_4 acid reaction of Dubois (Bendiak & Cook, 1983), a strong positive result was obtained. However examination of the unhydrolysed solution by paper chromatography stained by the silver staining technique of Trevelyan (Bendiak & Cook, 1983), failed to reveal the presence of any small hexoses (G. M. W. Cook & B. Bendiak, personal communication). It seems that whatever linkages were broken in the Sephadex gel produced very large saccharide units. Interestingly, the G-25 Sephadex which was used to desalt the enzyme

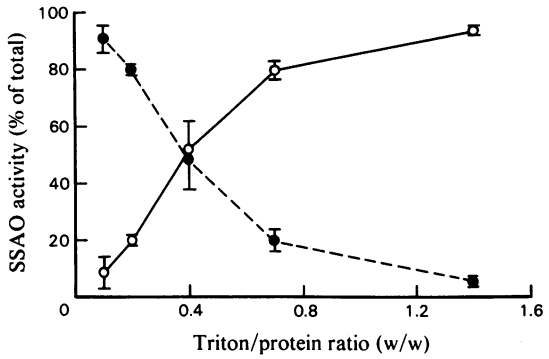

Fig. 1. *Solubilization of SSAO from brown adipose tissue membrane fractions by titration with Triton X-100*
Samples of membrane pellets containing 1mg of protein/ml were treated at 4°C with 0.01, 0.02, 0.04, 0.07 and 0.14% (w/v) Triton X-100 in 20mM-phosphate buffer, pH7.8, and centrifuged at 100000*g* for 1h. Supernatant (○) and pellet (●) samples were assayed in quadruplicate for SSAO activity with 0.025mM-benzylamine as substrate. Each point is the mean (±S.E.M.) of between three and six separate experiments.

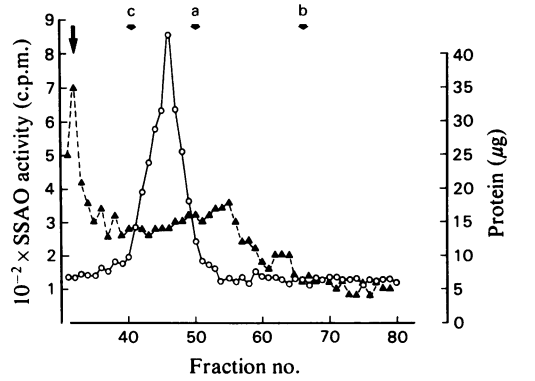

Fig. 2. *Gel filtration profile of SSAO solubilized from brown adipose tissue membranes*
SSAO solubilized with 0.08% Triton X-100 was loaded on to a calibrated column of Ultrogel AcA 34 and eluted with 20mM-phosphate buffer, pH7.8, containing 0.1% Triton X-100. Each fraction (0.96ml) was assayed in quadruplicate for SSAO activity (○) with 0.025mM-benzylamine as substrate and for protein (▲) by the method of Lowry *et al.* (1951). The total enzyme activity recovered was over 80% of that applied to the column. Arrows indicate positions of elution of void volume, and of catalase (c), aldolase (a) and bovine serum albumin (b). The bed volume (not shown) was at fraction 87.

preparation was not destroyed. It is possible that the degree of cross-linkage could explain this finding. G-25 is highly cross-linked, whereas G-200 has only a small number of linkages.

When enzyme solubilized by 0.08% (w/v) Triton and partially purified by gel filtration was subjected to affinity chromatography either with agarose-bound concanavalin A or with agarose-bound lentil lectin, over 80% of the enzyme activity became bound even after extensive washing. Elution of this activity was attempted initially with 0.25M-α-methyl D-mannoside. Although between 50 and 60% of SSAO activity could be eluted from the *Lens culinaris* lectin by these means, only 10–20% could be recovered from the Glycosylex A resin. It is known that the lectin from *Lens culinaris* binds the same sugar residues as does concanavalin A but its affinity is 50 times lower (Stein *et al.*, 1971; Goldstein, 1976). Later, elution of SSAO activity from the lentil lectin was undertaken by using a continuous gradient from 0 to 0.5M-mannoside (Fig. 3).

The affinity of SSAO for its substrate, benzylamine (Table 1), was found to be unchanged by solubilization. The order of potency of inhibition of benzylamine deamination by the competitive substrates, phenylethanolamine, phenylethylamine, tyramine and octopamine was similar with both forms of the enzyme. The soluble enzyme also showed a similar sensitivity to inhibition by cuprizone and was still unaffected by cyanide and β-aminopropionitrile at 10^{-4}M. The copper-chelating agent, diethyldithiocarbamate, however, used at concentrations as high as 10^{-3}M did not affect the activity of either the soluble form of SSAO or the membrane-bound enzyme and the thiol reagent *p*-chloromercuriphenylsulphonate caused reversible inhibition of SSAO at 10^{-3}M only, but not at lower concentrations.

By solubilization with 0.08% (w/v) Triton, gel filtration and affinity chromatography, it was possible to purify the enzyme about 90-fold (Table 2).

The M_r of SSAO, still bound to the membrane, was determined by the technique of irradiation inactivation. Freeze-dried samples of brown fat homogenates, of 'tissue membranes' and of SSAO preparations solubilized with 0.08% Triton were exposed to doses of radiation up to 12Mrad. Preliminary experiments established that the process of freeze-drying and of warming the freeze-dried samples to 60°C for several minutes (about

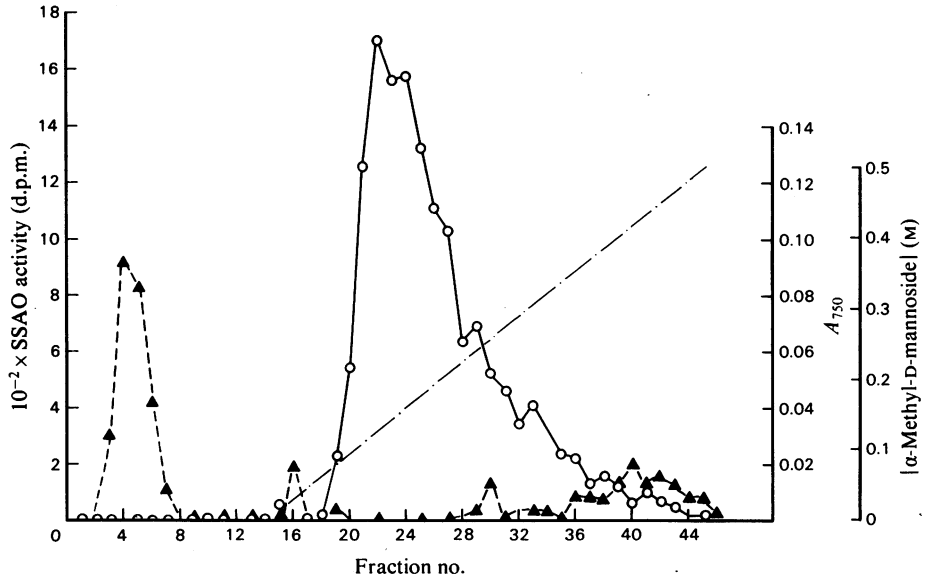

Fig. 3. *Affinity chromatography profile of solubilized SSAO on a column of Lens culinaris lectin*
Solubilized SSAO, partially purified by gel filtration, was loaded on to a column of *Lens culinaris* lectin immobilized on agarose beads, equilibrated with 20mM-phosphate buffer, pH7.8, containing 0.1% Triton X-100, 0.1mM-$MgCl_2$, 0.1mM-$CaCl_2$, 0.1mM-$MnCl_2$ and 0.5M-NaCl and washed with 10 column volumes of the buffer (fractions 1–10). Then 5 column volumes of 20mM-phosphate buffer containing 0.01M-EDTA were passed down the column (fractions 11–14) followed by a continuous gradient from 0 to 500mM-α-methyl D-mannoside (–·–·–) in the buffer/EDTA (fractions 15–45). Aliquots (0.5ml) of the eluate were collected at all stages and assayed in quadruplicate for SSAO activity (○) and for protein (▲) by the method of Lowry *et al.* (1951). Over 50% of the enzyme activity and over 85% of the protein applied was recovered.

Table 2. *Purification of SSAO by Triton X-100 solubilization, gel filtration and affinity chromatography*
Results shown are from one of four separate preparations undertaken. The SSAO activity of each sample was measured in quadruplicate with 0.025mM-benzylamine as substrate. Relative specific activity at each stage was calculated from % of total original enzyme activity recovered/% of total original protein recovered.

	SSAO				
	Specific activity (nmol/h per mg of protein)	Total activity (nmol/h)	Yield (%)	Protein yield (%)	Relative specific activity
Original homogenate	28.61	10009			
'Tissue membranes'	73.66	9234	92	36	2.56
0.08% Triton solubilization:					
Supernatant	113.71	7022	70	18	3.89
Pellet	3.04	206	2	19	0.11
Gel filtration pooled fractions	190.21	5153	51	8	6.62
Affinity chromatography pooled fractions	2373	2421	24	0.3	83.40

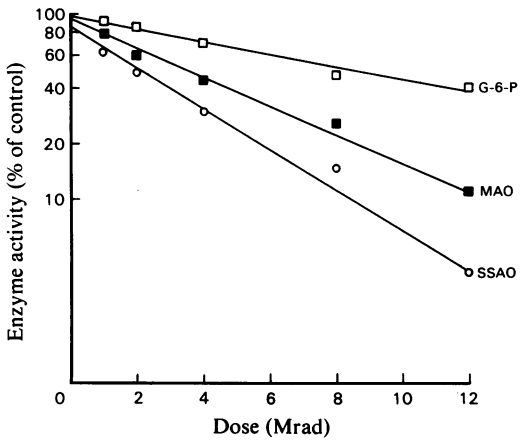

Fig. 4. *Irradiation inactivation analysis of SSAO*
Freeze-dried samples of brown adipose tissue membranes, some prepared in 1mM-Tris/HCl buffer, pH7.8, for glucose-6-phosphatase (G-6-P) (□) assay and for SSAO assay and some in 1mM-phosphate buffer, pH7.8, for MAO (■) and SSAO (○) assay, were irradiated under vacuum with 1, 2, 4, 8 and 12Mrad. After reconstitution with water, enzyme activity in each sample was assayed in quadruplicate and compared with the activity of non-irradiated samples. Data were analysed by linear regression and the slope, k, was used to calculate M_r with the empirically derived equation:

$$M_r = k \times 6.4 \times 10^{11} = \frac{6.4 \times 10^{11}}{D_{37}}$$

where D_{37} is the dose (in rads) at which 37% of the original activity remains. In the above experiment the M_r values of the three enzymes were calculated to be 52000 for glucose-6-phosphatase, 122000 for MAO and 186000 for SSAO.

the extent of the heating which would be caused by irradiation) did not significantly inactivate the enzyme. It was assumed that any loss of enzyme activity observed was therefore due entirely to the effect of irradiation. The reduction in activity of SSAO, MAO, and glucose-6-phosphatase after irradiation is shown in Fig. 4. As expected, the degree of inactivation in the form of the natural logarithm of the percentage of enzyme activity remaining was found to be linearly related to the dose of radiation. From the slopes of the regression lines it was possible to calculate the M_r of SSAO, after corrections had been made for variations in radiation administered using data from the internal standards. The M_r or functional unit size of MAO-A was taken to be 118000 (Parkinson, 1979) and that of glucose-6-phosphatase 70000 (Collipp *et al.*, 1974). Similar data for SSAO were obtained from membrane-bound, i.e. homogenates and tissue membranes, and from Triton-solubilized samples, and from samples in Tris and in phosphate buffer. The influence of buffer was checked in view of past observations which have shown a discrepancy in the size estimate for acetylcholinesterase with different buffers (Parkinson & Callingham, 1982). The M_r of SSAO was calculated to be 183000 ± 5000 (mean $\pm$ S.E.M.) by using data from eight experiments.

Discussion

The SSAO activity in tissue membranes of homogenates of the brown adipose tissue of rats was released into the supernatant by the use of the non-ionic detergent Triton X-100. Mild treatments, such as raising or lowering the ionic

strength of the surrounding medium or treating with chelating agents, procedures capable of removing the loosely bound extrinsic proteins by alteration in electrostatic interactions (Coleman, 1973; Reynolds, 1981) were without effect in the absence of added Triton. When this soluble fraction was subjected to gel filtration, the SSAO activity was recovered at a position corresponding to a M_r of between 160000 and 180000. When Triton concentration in this soluble fraction was decreased by about 80% the enzyme activity appeared in the void volume instead, suggesting that aggregation had occurred.

Depletion of the lipid content of particulate fractions of brown fat resulted in a considerable loss of SSAO activity, and a lowering of the affinity of the enzyme for its substrates. This would suggest that the lipid attachment of the enzyme to the cell membrane is of some importance for its activity. Although the efficacy of the extraction method in brown fat was not tested, butan-2-one, used as described, is known to remove 80% of total lipids from rat liver mitochondria (Ekstedt & Oreland, 1976), preferentially extracting phosphatidylcholine and phosphatidylethanolamine and leaving behind higher proportions of acidic phospholipids. Likewise, treatment of mouse liver mitochondria with acetone has been found to remove 80% of neutral phospholipids, the addition of ammonia releasing some of the acidic phospholipids as well (Tong *et al.*, 1979). There is evidence to show that phosphatidylethanolamine is predominantly found on the inside of plasma membranes of erythrocytes and some other cells, whilst acidic phospholipids occur mainly on the outside (Rothman & Lenard, 1977; Higgins & Evans, 1978). However, this mode of phospholipid asymmetry is in no way universal. Until the exact distribution of phospholipids within the brown fat cell membrane has been established this approach does not allow any firm conclusion about the membrane location of SSAO to be drawn.

The use of gel filtration, a technique only relevant to globular proteins, to determine the size of the enzyme–detergent complex may lead to erroneous estimations of the molecular mass of the enzyme (Reynolds, 1981). Membrane-bound proteins derive their three-dimensional structure by interaction with other components. Thus removal of the protein from this environment, e.g. by solubilization, can lead to significant changes in conformation. In particular glycoproteins may behave in an anomalous fashion on gel filtration. It was therefore important to establish whether or not similar values for the molecular mass of the SSAO could be obtained by other methods, for example irradiation inactivation analysis. This method is particularly useful for determining the size of the functional enzyme while still bound to the cell membrane. Bombardment of an enzyme with high energy electrons or other ionizing radiation causes progressive inactivation, the sensitivity of the enzyme to this inactivation being a function of molecular mass (Kepner & Macey, 1968). With this technique an M_r of about 183000 for the membrane-bound and for the solubilized SSAO was found. This is in good agreement with the value obtained by gel filtration and may indicate that the enzyme in the presence of Triton is roughly spherical. These values are also similar to those reported for ox and pig plasma amine oxidase, pig kidney histaminase, aorta and dental pulp amine oxidase (Yasunobu *et al.*, 1976).

Like both the ox and pig plasma amine oxidases (Yasunobu *et al.*, 1976; Falk *et al.*, 1983), SSAO appears to interact with concanavalin A and also with the lentil lectin, which has the same sugar specificity (Goldstein, 1976), indicating the presence on the enzyme of mannose- and glucose-containing residues. Pig plasma amine oxidase (Falk *et al.*, 1983) in fact shows a heterogeneity which appears to be based at least partly on differences in carbohydrate content. No such heterogeneity in SSAO has yet been identified. It is well established that all protein-bound carbohydrates in animal cell plasma membranes are located on the external surface (Rothman & Lenard, 1977). Several different lines of evidence suggest that the SSAO enzyme may be located at or within the plasma membrane (Barrand & Callingham, 1982; Barrand *et al.*, 1983*a*,*b*). It would seem therefore that at least part of the enzyme must face outwards. Whether the active site of the enzyme is also on the outside is not clear, since the presence of concanavalin A does not affect enzyme activity, suggesting that the carbohydrate-containing portion is probably situated at some distance from the active site.

In these studies it was found that the activity of membrane-bound SSAO could be inhibited by the thiol reagent *p*-chloromercuriphenylsulphonate, but only at 10^{-3} M and even then the reaction was reversible. Results of studies on other non-FAD-dependent amine oxidases have indicated that thiol groups may not be essential for activity (Yasunobu *et al.*, 1976). On the other hand, MAO is known to be inactivated by such reagents. Observations on the nature of the reaction with ox liver MAO suggest that two cysteine residues at the active site of the enzyme are required for activity (Gomes *et al.*, 1976).

It seems therefore that brown fat SSAO shares certain properties with the copper-containing amine oxidases that have been studied in plasma, particularly ox and pig, and in dental pulp; M_r of around 170000–180000, sensitivity to carbonyl

reagents, comparative insensitivity to thiol reagents, the presence of carbohydrate moieties and the substrate requirement for a primary amine structure. However, substrate specificity is different, SSAO, in the amounts present in tissue homogenates, being unable to metabolize diamines or polyamines (Barrand & Callingham, 1982). Also the presence of copper as a vital component of the enzyme is open to doubt. Although cuprizone has been shown to inhibit the enzyme in brown adipose tissue (Barrand & Callingham, 1982), this effect may be entirely due to its action as a carbonyl reagent. Diethyldithiocarbamate was without effect on SSAO activity in brown adipose tissue and it has been reported that SSAO in rat heart (Lyles *et al.*, 1983) and in rat skull (Andree & Clarke, 1981) is also insensitive to inhibition by another copper-chelating agent, penicillamine. Indeed the possible absence of copper as a vital component may explain why rat SSAO is not sensitive to inhibition by cyanide (Yamada & Yasunobu, 1962).

SSAO, unlike many of the copper-containing amine oxidases, is a membrane-bound enzyme probably located in the outer cell membrane. From its glycoprotein nature it seems likely that at least the carbohydrate moiety of the enzyme should be externally facing. But it is not yet clear on which side of the membrane the active site is to be found, although it does appear to be some distance from the carbohydrate part of the enzyme. If SSAO does turn out to be located in this manner, it raises interesting possibilities for its function. Although its natural substrate is still unknown, it could be that the enzyme can use an extracellular monoamine without the need for an uptake system such as that required by MAO. The products of the resulting deamination could then be employed either in or on the membrane or even inside the cell. The enzyme has a low capacity for the substrates so far examined (Barrand & Callingham, 1982; Callingham, 1982), so it seems unlikely to be a scavenger of deleterious amines. It is more attractive to believe that some product of its deaminating activity may be important. For example, preliminary observations (M. A. Barrand & B. A. Callingham, unpublished work) confirm that SSAO, like MAO, produces H_2O_2. MAO has been shown to have a possible role as a peroxide generator in the central nervous system, where it can influence the production of prostaglandins (Seregi *et al.*, 1982, 1983). In particular, H_2O_2 has been shown in adipocytes to act as a transmembrane messenger in polypeptide hormone action (Mukherjee & Mukherjee, 1982). It is important to try to discover whether or not SSAO is involved in transmembrane signalling or in some similar membrane activity.

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