Immunochemical Characterization of Monoamine Oxidase from Human Liver, Placenta, Platelets and Brain Cortex

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1. Antiserum raised to purified human liver monoamine oxidase was used to characterize the monoamine oxidase from human liver, brain cortex, placenta and platelets. 2. Antibodies to monoamine oxidase were purified by adsorption with a mitochondrial preparation. 3. Monoamine oxidase was present in liver particle-free supernatant as measured by enzyme activity and immunodiffusion. 4. Multiple precipitin lines were obtained on immunodiffusion analysis against the purified liver enzyme. It is proposed that this is due to either aggregation or to differential lipid binding. 5. The results suggest that the functionally different enzymes found in liver, brain cortex, platelets and placenta are immunochemically related and may be identical.

The immunochemical relationship of the different forms of monoamine oxidase, defined by substrate specificities, either within a single tissue or between several tissues or cell types of a single species has been the subject of several studies (Hidaka *et al.*, 1971; McCauley & Racker, 1973; Dennick & Mayer, 1977; Powell & Craig, 1977). Two of these investigations (McCauley & Racker, 1973; Powell & Craig, 1977) have shown the presence of two immunologically distinct forms in the tissues chosen for study. McCauley & Racker (1973) prepared an antiserum to bovine liver monoamine oxidase that did not cross-react with the monoamine oxidase present in bovine brain mitochondria that preferentially deaminates 5-hydroxytryptamine, but the antiserum did react with the monoamine oxidase in bovine brain mitochondria that preferentially deaminates benzylamine. Powell & Craig (1977) raised an antiserum to human placental monoamine oxidase that gave a single precipitin line on double-diffusion analyses against placental enzyme, but gave no reaction against human platelet enzyme. This antiserum also reacted with mitochondrial Triton X-100 extracts from IMR32 cells (human neuroblastoma line; Tumilowicz *et al.*, 1970), but not with mitochondrial Triton X-100 extracts of MC-63 cells (a HeLa derivative cell line; Siegel *et al.*, 1976).

In contrast, Hidaka *et al.* (1971) and Hartman *et al.* (1971) could find no antigenic differences between bovine brain monoamine oxidase and the liver enzyme or between the electrophoretically separable forms of liver monoamine oxidase with an antiserum raised to purified bovine liver monoamine oxidase. The antiserum to highly purified human liver monoamine oxidase prepared by Dennick &

Mayer (1977) was observed to immunoprecipitate completely the enzyme activities in Triton X-100 extracts of liver monoamine oxidase towards tyramine, β -phenethylamine and 5-hydroxytryptamine.

In the present paper a new antiserum prepared to purified human liver monoamine oxidase has been used to study the immunochemical relationship of putative antigens in Triton X-100 extracts of human liver mitochondria, brain-cortex non-synaptosomal and synaptosomal mitochondria, placental mitochondria, platelets and a particle-free supernatant prepared from liver. A preliminary report of this work has been published (Russell *et al.*, 1978).

Materials and Methods

Animals

Sheep were obtained from the Joint Animal Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. Human liver and brain cortex were obtained within 12 h of death from a 62-year-old male (accidental death) who had no history of psychiatric illness. The liver and brain cortex were kindly supplied by Dr. A. Stevens, Department of Pathology, University of Nottingham, Nottingham, U.K. The placenta was supplied by Dr. D. Craven, Department of Obstetrics and Gynaecology, City Hospital, Nottingham, U.K. Platelets were obtained from fresh human blood given by laboratory personnel with their informed consent.

Materials

Tyramine hydrochloride, benzylamine hydrochloride, 5-hydroxytryptamine (creatinine sulphate

Abbreviation used: IgG, immunoglobulin G.

complex), Coomassie Brilliant Blue R, 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-di-methoxy-4,4'-diphenylene)ditetrazolium chloride and glycine were purchased from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Triton X-100 was purchased from Lennig Chemicals Ltd., Croydon, Surrey CR9 3NB, U.K. Repelcote and CNBr were purchased from Hopkin and Williams Ltd., P.O. Box 1, Romford RM1 1HA, Essex, U.K. Sepharose 4B was purchased from Pharmacia (G.B.) Ltd., London W.5, U.K. The MSE sonicator was purchased from MSE Scientific Instruments, Manor Royal, Crawley, West Sussex, U.K. All other chemicals were of A.R. grade.

Purification of liver monoamine oxidase

Liver monoamine oxidase was purified as described in Dennick & Mayer (1977). The purified enzyme had a specific activity with tyramine of 3.15 nmol/mg of protein and had no measurable 5-hydroxytryptamine-oxidizing activity.

Preparation of Triton X-100 extracts of brain cortex, liver and placental mitochondria and platelets

Triton X-100 extracts of human liver mitochondria were prepared as described by Dennick & Mayer (1977). Brain-cortex non-synaptosomal and synaptosomal mitochondria were prepared as described by Whittaker (1969). Triton X-100 extracts were prepared by suspending the mitochondria in 20 mM-potassium phosphate, pH 7.2, containing 1.5% (w/v) Triton X-100 at a concentration of 10–15 mg/ml and sonicating for 2 min in an MSE sonicator.

Fresh placenta (100 g) was homogenized in an MSE Atomix homogenizer for 1 min with 3 vol. of 0.32 M-sucrose. All procedures were carried out at 4°C. The fibrous tissue was removed by centrifugation for 15 min at 1000 *g*_{av.}. The resulting supernatant was centrifuged at 10000 *g*_{av.} for 10 min to yield a mitochondrial pellet. The mitochondria were washed once with 0.32 M-sucrose and re-centrifuged. A Triton X-100 extract was prepared as for liver mitochondria (Dennick & Mayer, 1977).

Blood (100 ml) was collected in plastic syringes and transferred to siliconized glass centrifuge tubes containing 4.2 ml of 100 mM-Na₂EDTA, pH 7.0, as an anticoagulant. The solution was centrifuged for 10 min at 300 *g*_{av.} at room temperature and the upper yellow layer of platelet-rich plasma retained. The platelets were collected by centrifugation at 1500 *g*_{av.} for 15 min. The pellet was washed with 20 mM-sodium phosphate, pH 7.0, containing 0.15 M-NaCl and 100 mM-Na₂EDTA and centrifuged as before. The platelets were solubilized in 0.1 ml of 20 mM-sodium phosphate, pH 7.0, containing 0.15 M-NaCl and 1.5% (w/v) Triton X-100 after sonication for 30 s in an MSE sonicator.

Preparation of a particle-free supernatant from human liver

The supernatant remaining after the preparation of liver mitochondria was centrifuged for 6000000 *g*-min to yield a particle-free supernatant.

Preparation of antiserum

The antiserum used in these experiments is the same antiserum to human liver monoamine oxidase as that described in the preceding paper (Russell *et al.*, 1979).

Immunodiffusion analyses

Double-diffusion analyses (Ouchterlony, 1968) were carried out in 1% (w/v) agarose in 20 mM-potassium phosphate, pH 7.0, and 0.15 M-NaCl containing 1.5% (w/v) Triton X-100. Immunoprecipitin lines to be stained for protein were allowed to develop for 2 days at room temperature. Immunoprecipitin lines were stained with Coomassie Brilliant Blue as described by Mayer & Walker (1978), after washing to remove unprecipitated protein. Immunodiffusion gels to be stained for monoamine oxidase activity were incubated for 3 days at 4°C. The gels were washed in 20 mM-potassium phosphate (pH 7.0)/0.15 M-NaCl and pressed for 10 min as described by Mayer & Walker (1978). The gels were allowed to take up the staining solution composed of 5 mM-tyramine hydrochloride, 1.5 mM-Na₂SO₄, 0.3 mM-2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)ditetrazolium chloride in 25 mM-potassium phosphate buffer, pH 7.2, and were then incubated in the staining solution overnight at 37°C in the dark. Dark-blue staining of a precipitin line indicated the presence of monoamine oxidase activity. Control precipitin lines with an antigen-antibody system of casein-(casein antiserum) (Al-Sarraj *et al.*, 1978) mixed with monoamine oxidase preparations did not stain in this system.

Mitochondrial adsorption of the antiserum

Antiserum and liver mitochondria in 100 mM-potassium phosphate buffer, pH 7.2, were mixed in proportions of 2 mg of antiserum protein to 1 mg of mitochondrial protein and left at 4°C for 2–3 h. The suspension was centrifuged at 100000 *g*_{av.}-min and the resulting pellet washed twice with 20 mM-sodium phosphate containing 0.15 M-NaCl. The pellet was then treated with the same volume of 0.1 M-glycine/HCl, pH 2.8, as the original antiserum volume for 1 h at 0°C with constant stirring. The suspension was centrifuged for 225000 *g*_{av.}-min, and 1 M-potassium phosphate, pH 7.2, added to the supernatant to adjust the solution to pH 7.2. The solution was then dialysed overnight at room temperature against 20 mM-sodium phosphate, pH 7.0, containing 0.15 M-NaCl.

Protein determinations

Protein at concentrations of 5–30 mg/ml was determined by the method of Gornall *et al.* (1949) and at lower concentrations by the method of Lowry *et al.* (1951).

Enzyme assays

Monoamine oxidase was assayed with either the coupled spectrophotometric method of Houslay & Tipton (1973) with ox liver aldehyde dehydrogenase or the radiochemical method described in the preceding paper (Russell *et al.*, 1979). The spectrophotometric assays used tyramine hydrochloride (final concn. 2.3 mM) or benzylamine hydrochloride (final concn. 0.1 mM) or 5-hydroxytryptamine (final concn. 1.1 mM). The radiochemical assay used [2-¹⁴C]tyramine or 5-hydroxy[2-¹⁴C]tryptamine or β -phen[1-¹⁴C]ethylamine at 1 mM concentration and specific radioactivity of 5 mCi/mmol.

Preparation of (liver particle-free supernatant protein)–Sephacrose

Liver particle-free supernatant proteins were coupled to Sepharose-4B (10 mg of protein/ml of Sepharose 4B) after moderate CNBr activation of the Sepharose by the method of Porath *et al.* (1973).

Results

Fig. 1 shows the results obtained when liver particle-free supernatant, a Triton X-100 extract of liver mitochondria and purified liver monoamine oxidase were compared immunochemically with the Ouchterlony (1968) immunodiffusion technique. IgG prepared from the antiserum by the method described by Mayer & Walker (1978) is in the central well. A reaction of identity (Fig. 1a) was formed between the antiserum and antigen in all three preparations. A second precipitin line was also formed between the antiserum and the purified liver monoamine oxidase. The line of identity between the purified monoamine oxidase and the Triton X-100 extract of liver mitochondria could also be stained for monoamine oxidase activity (Fig. 1b) by using the histochemical-staining technique of Glenner *et al.* (1957). The particle-free supernatant was assayed for monoamine oxidase activity by using the radiochemical method. A specific activity towards tyramine of 0.7 nmol/mg of protein was determined.

Antibodies to monoamine oxidase were bound to (liver particle-free supernatant protein)–Sephacrose and could be eluted by 0.1 M-glycine/HCl, pH 2.8, as shown by the fact that IgG not binding to the adsorbent did not give a precipitin line with purified enzyme, whereas IgG that bound and was eluted did

Fig. 1. Immunodiffusion of liver particle-free supernatant (PFS), purified liver monoamine oxidase (PM), and Triton X-100 extracts of liver mitochondria (LTX) against antiserum (Ab)

The gel was stained for protein (a) and for enzyme activity (b). The staining procedures are given in the Materials and Methods section.

give a reaction of identity. No specific binding of anti-(monoamine oxidase) antibodies to Sepharose was observed. The immunoinhibition titre of the antiserum with liver monoamine oxidase was found to decrease after previous incubation of the antiserum with liver particle-free supernatant protein and centrifugation to remove any precipitate (Fig. 2).

Antibodies to monoamine oxidase were purified by mitochondrial adsorption of the antiserum (described in the Materials and Methods section). Protein bound by the mitochondria (4% of antiserum protein) and eluted with 0.1 M-glycine/HCl, pH 2.8, retained 60% of the immunoinhibitory capacity of the non-adsorbed antiserum. The mitochondrially adsorbed antiserum was used to compare liver particle-free supernatant, pure liver monoamine oxidase and Triton X-100 extract of liver mitochondria (Fig. 3).

A reaction of identity was formed with all three preparations. Two additional precipitin lines were formed between the antiserum and purified liver monoamine oxidase. A faint secondary precipitin line was formed by both the liver particle-free supernatant and the Triton X-100 extract of liver mitochondria.

Fig. 2. Effect of particle-free supernatant on immunoinhibition capacity of antiserum

Antiserum (150 μ l) was mixed with increasing amounts of freeze-dried particle-free supernatant protein (0–1.5 mg) and incubated overnight at 4°C. The antiserum samples were centrifuged at 14000 g_{av} for 6 min and the supernatant (20 μ l) incubated with samples of Triton X-100 extract of liver mitochondria (20 μ l) overnight at 4°C. The samples were assayed radiochemically for monoamine oxidase activity with tyramine and the immunoinhibition (%) calculated.

The substrate specificities of Triton X-100 extracts of placental mitochondria, liver mitochondria and brain-cortex non-synaptosomal and synaptosomal mitochondria are shown in Table 1. The total monoamine oxidase activity of the platelet extract was so low that after removal of the bulk (80%) of the enzyme for immunodiffusion analysis, insufficient remained for measurement of substrate specificities. Fig. 4 shows the results obtained when Triton X-100 extracts of placental mitochondria, liver mitochondria, brain-cortex non-synaptosomal and synaptosomal mitochondria, platelets and purified liver monoamine oxidase were compared on Ouchterlony immunodiffusion analyses against mito-

Fig. 3. Immunodiffusion of liver particle-free supernatant (PFS), purified liver enzyme (PM) and Triton X-100 extract of liver mitochondria (LTX) against mitochondrially adsorbed antiserum (Adsorbed Ab)

The gel was stained for protein with Coomassie Blue (see the Materials and Methods section).

Table 1. Substrate specificities of brain-cortex, liver and placental monoamine oxidase

Monoamine oxidase activities in placental and liver mitochondrial preparations were measured with the coupled spectrophotometric assay with aldehyde dehydrogenase (Houslay & Tipton, 1973). Monoamine oxidase activities in brain mitochondrial preparations were measured with the radiochemical assay as described in Tipton & Youdim (1976).

Preparation	Substrate	Activity (nmol/min per mg of protein)	Ratio
Placenta	Tyramine	19.9	1
	Benzylamine	4.6	0.23
	5-Hydroxytryptamine	22.2	1.11
Liver	Tyramine	4.5	1
	Benzylamine	11.7	2.57
	5-Hydroxytryptamine	0.8	0.17
Brain cortex Non-synaptosomal	Tyramine	1.3	1
	β -Phenethylamine	2.3	1.77
	5-Hydroxytryptamine	0.3	0.23
Synaptosomal	Tyramine	1.5	1
	β -Phenethylamine	2.5	1.67
	5-Hydroxytryptamine	0.1	0.07

Fig. 4. Immunodiffusion of human tissue extracts and purified liver monoamine oxidase against mitochondrially adsorbed antiserum (Ab)

The Triton X-100 extracts were: placental (PLAC), liver (LTX), non-synaptosomal (NS) and synaptosomal (S) mitochondria, platelets (PLAT) and particle-free supernatant (PFS). The gel was stained for protein with Coomassie Blue (see the Materials and Methods section).

chondrially adsorbed antiserum. A reaction of identity was formed with all samples. A second line of antigenic identity was also seen in samples of Triton X-100 extracts of liver and brain-cortex non-synaptosomal mitochondria, liver particle-free supernatant and purified liver monoamine oxidase. The precipitin line of identity to Triton X-100 extracts of liver and non-synaptosomal mitochondria, platelets and purified liver monoamine oxidase could also be stained for monoamine oxidase activity (results not shown). The number of precipitin lines formed on immunodiffusion analysis with mitochondrially adsorbed antiserum and Triton X-100 extracts of liver mitochondria, placental mitochondria and purified liver monoamine oxidase increased with the length of time (0–5 months) the samples were stored at -20°C .

Discussion

To use an antiserum in a study of the immunochemical relationship of a purified antigen and putative antigen in extracts of liver, brain cortex, placenta and platelets, it is essential to ensure that the antiserum is monospecific for the antigen (i.e. monoamine oxidase) (Walker *et al.*, 1976; Mayer & Walker, 1978). Highly purified liver monoamine oxidase was used to raise the antiserum to minimize the possibility of producing antibodies to contaminating antigens (Russell *et al.*, 1979). The antiserum was tested against fresh Triton X-100 extract of liver mitochondria and liver particle-free supernatant to detect any contaminating antibodies other than anti-(monoamine oxidase) antibodies

(Fig. 1). Triton X-100 extract of liver mitochondria was chosen since it contains putative contaminating membrane antigens that could be present in the purified enzyme, and liver particle-free supernatant was chosen since it contains putative soluble contaminating antigens. A single precipitin line having antigenic identity with the purified enzyme was formed by both preparations. The precipitin lines to purified enzyme and the Triton X-100 extract of liver mitochondria could be stained for monoamine oxidase activity (Fig. 1b).

Monoamine oxidase activity was found in the particle-free supernatant (0.7 nmol/mg of protein). Also, (particle-free supernatant protein)-Sephacrose selectively bound anti-(monoamine oxidase) antibodies, and particle-free supernatant immunoprecipitated anti-(monoamine oxidase) antibodies (Fig. 2) from the antiserum. This is considered conclusive evidence that the liver particle-free supernatant contains monoamine oxidase and therefore explains the immunoprecipitin line formed with the antiserum (Figs. 1 and 3). Since monoamine oxidase is known to be located in the mitochondrial outer membrane (Ernster & Kuylenskierna, 1970), some enzyme may be dislodged from the outer membrane during the preparation of the particle-free supernatant. Alternatively enzyme in the 'particle-free' supernatant may be microsomal in origin (de Duve & Baudhuin, 1966). The additional precipitin line formed between the purified monoamine oxidase and the antiserum (Fig. 1) was not present in the Triton X-100 extract of liver mitochondria from which the enzyme had been prepared. Multiple lines may be due to different amounts of lipid binding (Hackenbrock & Hammon, 1975), aggregation, or to proteolytic modification of the enzyme (Bjerrum & Bøg-Hansen, 1976). It is unlikely that the multiple precipitin lines were due to slight variations in temperature during diffusion (Leisgang bands; Crowle, 1961), as multiple lines were observed in plates incubated at 4°C for 4 days and stained for protein with Coomassie Blue (results not shown). The multiple lines obtained on immunodiffusion analysis increased in number after storage of the samples (0–5 months) at -20°C (Figs. 3 and 4). Mitochondrial adsorption of the antiserum did not eliminate the incidence of multiple immunoprecipitin lines. Indeed, a third precipitin line was evident on analysis of purified liver monoamine oxidase with mitochondrially adsorbed antiserum (Fig. 3). There is evidence both for the existence of monoamine oxidase forms with different amounts of bound lipid (Houslay & Tipton, 1973) and for the existence of macromolecular aggregates of monoamine oxidase (Tipton, 1973). A single protein band was seen on electrophoresis of the purified enzyme in the presence of sodium dodecyl sulphate. This argues against any large-scale proteolytic action on the samples (Russell *et al.*, 1979).

The immunochemical analysis of monoamine oxidase in the Triton X-100 extracts of placental mitochondria, platelets, non-synaptosomal mitochondria, synaptosomal mitochondria and purified liver monoamine oxidase showed that all samples contained antigenically identical monoamine oxidase. The substrate specificities of the samples varied widely (Table 1), placental enzyme having a high ratio of 5-hydroxytryptamine oxidation to benzylamine oxidation, and liver enzyme having a high ratio of β -phenylethylamine oxidation to 5-hydroxytryptamine oxidation. There is evidence that platelet enzyme has a relatively low rate of oxidation of 5-hydroxytryptamine and a high rate of oxidation of β -phenylethylamine (Collins & Sandler, 1971).

The results suggest that functionally different enzymes (e.g. placental and platelet) are immunochemically identical. The data shown here and that of Dennick & Mayer (1977) support the contention of Houslay & Tipton (1973) that the multiple forms of the enzyme are due to modification of a single protein species by differential phospholipid binding.

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