Human Placental Diamine Oxidase

IMPROVED PURIFICATION AND CHARACTERIZATION OF A COPPER- AND MANGANESE-CONTAINING AMINE OXIDASE WITH NOVEL SUBSTRATE SPECIFICITY

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(Received 7 January 1976)

1. Isoelectric focusing studies on human placental diamine oxidase showed the pI value of the active enzyme to be 6.5. This information was used in modifying the enzyme purification by incorporating column chromatography on DEAE-Sephadex with ionic strength and pH-gradient elution and this, together with affinity chromatography on concanavalin A-Sephadex, gave a highly purified preparation, with a specific activity of 7.0 units/mg. 2. The enzyme gave the expected stoichiometry with *p*-dimethylaminomethylbenzylamine as substrate (K_{eq} , 2700) and also oxidized [8-arginine]vasopressin, [8-lysine]vasopressin, collagen and tropocollagen. Polyacrylamide gel slices showed identical migration of diamine-oxidizing and [8-lysine]vasopressin-oxidizing activity. 3. The molecular weight, determined by ultracentrifugation, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, variable polyacrylamide-gel electrophoresis and Sephadex G-200 column chromatography, was estimated to be approx. 70000. 4. E.s.r. spectroscopy showed that copper and manganese were present in the purified enzyme. This result was confirmed by atomic absorption spectroscopy, which indicated a stoichiometry for copper and manganese of approx. 1.0 and 1.2 g-atom respectively/70000 mol.wt. unit. 5. The e.s.r. spectral intensity did not decrease nor did the spectral line shape change when excess of *p*-dimethylaminomethylbenzylamine was added to the enzyme. 6. Addition of $K^{13}CN$ to the enzyme eliminated the copper e.s.r. signal without affecting the manganese signal. 7. The placental enzyme therefore appears to differ from other amine oxidases in terms of its metal cofactor requirement, molecular weight and substrate specificity, and possible roles *in vivo* for this enzyme are discussed.

Amine oxidases are enzymes that oxidize amines according to the stoichiometry:

The enzymes isolated from biological sources have tended to fall into one of two classes, those sensitive to thiol-group reagents, which oxidize monoamines well and diamines weakly or not at all [EC 1.4.3.4, amine-oxygen oxidoreductase (deaminating) (flavin-containing)], and those sensitive to carbonyl group reagents, which oxidize diamines preferentially over monoamines [EC 1.4.3.6, amine-oxygen oxidoreductase (deaminating) (pyridoxal-containing)]. The enzyme isolated from pig kidney (Bardsley *et al.*, 1971, 1973) appears to be a classical diamine oxidase, having little or no monoamine oxidizing ability, whereas the enzyme we have isolated from human placenta (Bardsley *et al.*, 1974*b*; Crabbe & Bardsley, 1974) does not belong precisely in either class, which is also the case for unpurified

pregnancy plasma diamine oxidase. This enzyme has now been purified (Baylin & Margolis, 1975) and shows very similar catalytic properties to the unpurified enzyme. We have investigated the physical characterization of the human placental diamine oxidase further in order to clarify its position in enzyme classification and this paper presents novel structural information and catalytic properties that place this enzyme definitively in a class of its own, separate from both classical mono- and di-amine oxidases.

Materials and Methods

Materials

Concanavalin A-Sephadex was from Pharmacia, London W.5, U.K.; Chelex 100 chelating resin was from Bio-Rad laboratories, St. Albans, Herts., U.K.; $K^{13}CN$ was a gift from Dr. R. C. Bray. [8-lysine]-

Vasopressin and [8-arginine]vasopressin were from Sigma (London) Chemical Co., Kingston-upon-Thames, KT2 7BH, U.K. All other purchased reagents were of AnalaR quality.

Spectrophotometric assays

(a) Diamine oxidase activity was determined by the direct spectrophotometric method by using *p*-dimethylaminomethylbenzylamine as substrate (Bardsley *et al.*, 1973).

(b) The oxidation of [8-lysine]vasopressin, [8-arginine]vasopressin, collagen, tropocollagen and other substrates was determined either by coupled oxidation with H_2O_2 by using 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) as chromogen (Bardsley *et al.*, 1974b; Childs & Bardsley, 1975) or by O_2 uptake measurements. One unit of enzyme activity is defined as that amount of enzyme catalysing the oxidation of $1\mu\text{mol}$ of substrate/min, at 20°C , and in 50mM-sodium/potassium phosphate buffer, pH7.0.

Isoelectric focusing

Electrofocusing was performed with the LKB Multiphor Isoelectric Focusing equipment and

polyacrylamide-gel rods, with 40% Ampholines giving pH ranges of 2–10 and 5–8. The results of a typical experiment with partially purified human placental diamine oxidase are shown in Fig. 1, over the pH range 5–8, where the active enzyme concentrates at pH6.5, as determined by duplicate experiments. A similar result was obtained in 6M-urea, indicating that any dissociable subunits have similar pI values.

Enzyme purification

This was substantially as described previously (Bardsley *et al.*, 1974b) except for the following amendments.

(a) *DEAE-Sephadex column chromatography using pH gradient elution.* After chloroform/ethanol (1:3, v/v) extraction, the supernatant was applied to a column (5.5cm $\times$ 60cm) of DEAE-Sephadex A-50, and the column eluted with a linear gradient of 0.02–0.1M-sodium/potassium phosphate buffer, pH7.2 and then with a linear gradient of 0.1M-phosphate buffer, pH7.2–6.0, by using an LKB Ultragrad gradient mixer (Fig. 2). The active fractions were pooled, concentrated and either applied directly to a concanavalin A-Sepharose column or first to a Sephadex G-150 column.

Fig. 1. Gel electrofocusing of partially purified human placental diamine oxidase

Determination of pH, amount of protein (by spectrophotometric determination) and enzyme activity were by elution of gel slices into water or sodium/potassium phosphate buffer, pH7.0, for 24h, followed by measurement of the required parameter. $\circ$, pH; $\square$, protein concentration; $\triangle$, enzyme activity.

Fig. 2. DEAE-Sephadex column chromatography of human placental diamine oxidase

○, Protein concentration (E_{280}); □, enzyme activity; ----, pH of elution buffer; ····, concentration of elution buffer.

Fig. 3. Concanavalin A-Sepharose 4B column chromatography of human placental diamine oxidase

○, Protein concentration (E_{280} , 1 cm path length); △, E_{408} (1 cm path length); □, enzyme activity. Enzyme was eluted with 0.1 mM- α -methyl glucoside at the point shown by an arrow.

(10cm×1 cm) of concanavalin A-Sepharose, equilibrated with 50mM-phosphate buffer, pH 7.0, containing 0.4M-NaCl to prevent non-specific adsorption. Enzyme was eluted with 0.1 mM- α -methyl glucoside (Fig. 3) and the active fractions (specific activity 6.0–7.0 units/mg) concentrated and stored at -15°C . A typical purification procedure is summarized in Table 1.

Stoichiometry

The rate of O_2 consumption was measured by using a Clark oxygen electrode connected to a specially modified Radiometer Blood System Analyser and Pye Unicam chart recorder. The system was calibrated with air-saturated (0.291 mm) water at 20°C and O_2 -free water.

Polyacrylamide-gel electrophoresis

This was carried out by using a modification of the method of Davis (1964). For enzyme determinations, extruded gels were cut into 1 mm slices with a microtome freezer, eluted into 50mM-phosphate buffer, pH 7.0, over 24 h at 4°C and assayed for enzyme activity spectrophotometrically.

Determination of equilibrium constant

The equilibrium constant for the reaction was measured in a Thünberg cuvette containing sodium/potassium phosphate buffer (pH 7.15, 1 0.22M), 50mM- NH_4Cl , 0.1 mM-*p*-dimethylaminomethylbenz-

(b) Concanavalin A-Sepharose affinity chromatography. After concentration with a Millipore ultrafilter, the enzyme was applied to a column

Table 1. *Purification of human placental amine oxidase*

For details see the text.

Step	Total vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
1. Initial homogenate from four placentae	700	70000	25	2.14×10^{-4}	1.0	100
2. Supernatant after chloroform/ethanol precipitation	650	1807	15	8.3×10^{-5}	39	60
3. DEAE-Sephadex chromatography	10	90	9	0.1	4.6×10^2	36
4. Affinity chromatography	4	1.2	8.4	7.0	3.08×10^4	33

aldehyde, KN_3 (5 mM, to inhibit any residual catalase activity causing bubble formation) and 0.04 unit of enzyme in a volume of 2 ml. H_2O_2 (40 μl ; 80 mM) was placed in the side arm (to give a final concentration of 1.6 mM), and the cuvette evacuated and equilibrated with 0.1 % O_2 . E_{250} was followed against a reference cuvette made up as above, but without NH_4Cl . The progress of the reaction was followed after mixing of the cuvette contents; from the final decrease in absorbance after 25 min, the equilibrium constant was calculated, assuming an ϵ_{250} of $10.4 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for *p*-dimethylaminomethylbenzaldehyde.

Molecular weight determination

(a) Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was used as described by Weber & Osborn (1969) with β -galactosidase, bovine serum albumin, ovalbumin, trypsin and pepsin as standards.

(b) Polyacrylamide-gel electrophoresis with various acrylamide concentrations (4–10%) was used (Hedrick & Smith, 1968).

(c) Gel chromatography on Sephadex G-200 (Andrews, 1965) was used with cytochrome *c*, trypsin, pepsin, ovalbumin, haemoglobin, bovine serum albumin and lactoperoxidase as standards. The void volume of the column (70 cm $\times$ 2 cm) was determined with Dextran Blue.

Analytical ultracentrifugation

Sedimentation-equilibrium studies were made by using the meniscus-depletion method with a double-sector cell, at 20°C on a Beckman model E analytical ultracentrifuge. Samples for ultracentrifugation were prepared in 50 mM-phosphate buffer, pH 7.0; for the sedimentation-equilibrium run under dissociating conditions, the sample was prepared by dialysis overnight at 4°C against 6 M-guanidinium chloride in 50 mM-phosphate buffer, pH 7.0.

Electron-spin-resonance (e.s.r.) spectroscopy

Samples for e.s.r. spectroscopy were passed through a small column (6 cm $\times$ 1 cm) of Chelex 100 resin

to remove any extraneously bound metal ions, then vacuum-dialysed to a concentration of approx. 50 mg/ml. Control experiments showed that Chelex 100 treatment of the enzyme had no effect on its enzymic activity.

X-band (9 GHz) spectra were run on a Decca X1 spectrometer (Decca, London, U.K.) in conjunction with a Varian 23 cm magnet and a Varian Fieldial magnetic-field sweep unit. The magnetic field was calibrated with a proton-resonance probe (Newport Instruments, Newport Pagnell, Northants., U.K.) and the microwave frequency with a wavemeter (Decca). An aqueous sample cell was used for the room temperature measurements; low-temperature spectra used the Decca variable-temperature accessory.

Q-band (35 GHz) spectra were run on a Varian V-4500 series spectrometer fitted with a low-temperature assembly for cooling the cavity. The magnetic field was calibrated with a proton-resonance probe as described above for the X-band measurements.

Quantitative determination of the copper and manganese content of the enzyme was made by using the integration procedure of Wyard (1965) with 2 mM- Cu^{2+} in 25 mM-EDTA (disodium salt) as standard. The copper and manganese content of the enzyme was confirmed by atomic-absorption analysis.

Results

Reaction stoichiometry and catalytic properties of the enzyme

Fig. 4(a) shows the rate of *p*-dimethylaminomethylbenzaldehyde production (and thus *p*-dimethylaminomethylbenzylamine consumption) plotted against the rate of O_2 consumption for highly purified human placental diamine oxidase, showing a 1:1 relationship between reactants and products.

Fig. 4(b) shows that the rate of O_2 consumption with *p*-dimethylaminomethylbenzylamine as substrate is halved on addition of catalase, which catalyses the reaction

and on further addition of 1mM-NaN₃, a specific catalase inhibitor, the initial reaction rate is restored. These data support the reaction stoichiometry

catalysed by the human placental amine oxidase. Attempts at NH₃ determination by a coupled assay using glutamate dehydrogenase (Lorenz *et al.*, 1967) proved unsuccessful owing to the non-linear kinetics of the assay system. (M. J. C. Crabbe, unpublished work). The equilibrium constant (K_{eq}) of the reaction was determined from the following concentrations of substrates and products present at equilibrium: *p*-dimethylaminomethylbenzylamine, 0.2 μM ; O₂, 14.6mM; *p*-dimethylaminomethylbenzaldehyde, 0.1mM; NH₄⁺, 50mM; H₂O₂, 1.6mM. The value of K_{eq} at 298 K was found to be 2700 giving a value for $\Delta G_0'$ of -19 kJ/mol.

In view of the suspected lysyl oxidase activity of this enzyme (Crabbe *et al.*, 1975), the oxidation of [8-lysine]vasopressin, [8-arginine]vasopressin, collagen, tropocollagen and collagenase-digested collagen (purified from rat tail by Dr. F. Steven, Department of Medical Biochemistry, University of Manchester) was investigated by O₂ consumption and coupled peroxidase assay by using 2,2'-azino-di-(3-ethylbenzthiazoline sulphonic acid) as chromogen. Fig. 5(a) shows double-reciprocal plots for *p*-dimethylaminomethylbenzylamine and [8-lysine]-vasopressin as substrate, the reaction being monitored with an oxygen electrode. K_m values of 10 μM and 18 μM respectively were determined by extrapolation of the linear portions of Fig. 5(a). The rates of oxidation of other substrates relative to *p*-dimethylaminomethylbenzylamine (0.5mM) were: [8-lysine]-vasopressin (0.5mM), 32%; [8-arginine]vasopressin (0.5mM), 50%; collagen (0.5mM), 40%; tropocollagen (0.5mM), 50%; collagenase-digested collagen (0.5mM), 85%. These determinations were made by both coupled assay and by using the oxygen electrode. Purified pig kidney diamine oxidase did not oxidize any of these substrates significantly. To investigate whether the lysyl oxidase activity was associated with the diamine-oxidizing activity, sliced polyacrylamide gels were assayed by using both spectrophotometric assays, as shown in Fig. 5(b) and 5(c), with partially purified and highly purified enzyme respectively. In

both cases, diamine-oxidizing activity and [8-lysine]-vasopressin-oxidizing activity was associated with the same band on polyacrylamide-gel electrophoresis.

Subunit composition and molecular weight

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis gave a mol.wt. estimate of 70000 for a single polypeptide chain; no further subunit dissociation was observed. Fig. 6(a) shows the elution behaviour of the enzyme on Sephadex G-200 relative to molecular weight standards; active enzyme eluted at a volume equivalent to a mol.wt. of 60000. Fig. 6(b) illustrates the results obtained with polyacrylamide gels of various concentrations and a replot of this graph (Fig. 6c) gives a mol.wt. for the active enzyme of 69500.

Sedimentation-equilibrium measurements on the native enzyme indicated a single species (the log c versus r^2 plot being approximately linear throughout) of mol.wt. 235000. The mol.wt. of the subunit produced under dissociating conditions was 82000. In these molecular weight calculations, a value for $\bar{v}$ of 0.725 was assumed.

Metal cofactors

E.s.r. spectroscopy of the partially purified enzyme revealed the presence of iron, presumably from contaminating haem proteins, and obscured the detection of manganese. The Q-band e.s.r. spectrum obtained from a highly purified sample (approx. 77% pure according to scanned sodium dodecyl sulphate gels) of the enzyme is shown in Fig. 7 and strongly suggests that Cu²⁺ and Mn²⁺ are tightly bound to the enzyme. Loosely bound metal ions would have been removed by the Chelex 100 column treatment. The measured e.s.r. parameters are: Cu²⁺, $g_{\perp} = 2.05$; Mn²⁺, $g_{\text{mean}} = 1.998$, $A_{\text{mean}} = 10.3\text{ T}$. Microwave-power-saturation studies suggest that the manganese species saturates more readily than the copper species. Addition of a tenfold excess of *p*-dimethylaminomethylbenzylamine did not significantly affect either the spectral intensity or the line shape. However, further addition to this same sample of K¹³CN, which might have been expected to

Fig. 4. Stoichiometry of the human placental amine oxidase reaction

(a) The rate of *p*-dimethylaminomethylbenzaldehyde production was measured as a function of O₂ consumption. Reaction mixtures containing various enzyme concentrations were assayed both spectrophotometrically and by the oxygen electrode. (b) O₂ consumption of human placental diamine oxidase (1) on addition of 0.4 mM-*p*-dimethylaminomethylbenzylamine, (2) further addition of 0.1 ml of catalase (5 mg/ml), (3) further addition of 1 mM-NaNO₃. The slopes of the different parts of the trace are given on the Figure.

cause superhyperfine splitting of the copper and/or the manganese signals through binding to the metal, in fact eliminated the copper signal (Fig. 8). This suggests that CN⁻ labilizes the Cu(II) centre in the enzyme and then reduces it to the Cu(I) state.

Integration of the e.s.r. spectrum against a copper

standard suggested an approximate copper and manganese stoichiometry of 0.7 and 0.3 g-atom/70000 mol.wt. unit respectively. This result was confirmed by atomic-absorption analysis, which revealed 1.0 g-atom of copper and 1.2 g-atom of manganese/70000 mol.wt. unit.

Fig. 5. [8-lysine]Vasopressin-oxidizing activity of human placental diamine oxidase

(a) Double-reciprocal plot for human placental diamine oxidase with, ○, *p*-dimethylaminomethylbenzylamine and, Δ, [8-lysine]vasopressin as substrates. Both compounds were assayed by measuring O_2 consumption. (b) and (c) Isolation of, ○, *p*-dimethylaminomethylbenzylamine-oxidizing activity and, Δ, [8-lysine]vasopressin-oxidizing activity from sliced polyacrylamide gels of (b) partially purified enzyme (after step 2, Table 1) and (c) highly purified enzyme (after step 4, Table 1).

Fig. 6. Molecular weight determinations

(a) Upward flow elution from Sephadex G-200 column with: (1) cytochrome *c*; (2) trypsin; (3) pepsin; (4) ovalbumin; (5) haemoglobin; (6) bovine albumin; (7) lactoperoxidase. Dextran Blue was used to calculate the exclusion volume. Active human placental diamine oxidase was eluted at the point indicated by an arrow. (b) Plot of protein mobility at various polyacrylamide-gel concentrations with: ○, human placental diamine oxidase; Δ, bovine serum albumin; □, haemoglobin; ●, lactoperoxidase; ▲, ovalbumin. (c) Replot of slopes from (b) as a function of molecular weight. Human placental diamine oxidase (arrow) gave a mol.wt. of 68 500.

Fig. 7. Q-band e.s.r. spectrum of placental diamine oxidase at 200 K

The enzyme concentration was 0.44 mM.

Fig. 8. Q-band e.s.r. spectrum of placental diamine oxidase

The final concentration of the enzyme was 0.53 mM. The spectrum was run at 200 K, following treatment with ^{13}KCN (final concn. 9.4 mM).

Discussion

The amine oxidases appear to have minimum mol.wt. units of approx. 90000, and most are dimers of mol.wt. approx. 200000. Evidence in support of this has been provided for the enzymes from pig kidney (Yamada *et al.*, 1967; Mondovi *et al.*, 1967), pig plasma (Buffoni & Blaschko, 1964; Boden *et al.*, 1973), bovine plasma (Achee *et al.*, 1968) and chick aorta (Siegal *et al.*, 1970). Exceptions to this occur with enzymes from pea seedling (mol.wt. 96000; Hill & Mann, 1964) and *Aspergillus niger* (mol.wt. 252000; Yamada *et al.*, 1968).

There is convincing evidence for the presence of bound Cu^{2+} as cofactor in the enzymes from pig kidney (Mondovi *et al.*, 1967), pea seedling (Hill & Mann, 1964), tobacco root (Mizusaki *et al.*, 1972), pig plasma (Buffoni & Blaschko, 1964; Buffoni *et al.*, 1968), bovine plasma (Yamada & Yasunobu, 1962),

Aspergillus niger (Yamada *et al.*, 1968) and chick aorta (Siegal *et al.*, 1970). It has also been found that all amine oxidases, except that from chick aorta, fail to oxidize lysyl peptides or collagen precursors; this observation has been confirmed in the present study with the diamine oxidase from pig kidney.

The results of the present investigation indicate that human placental diamine oxidase differs from most other amine oxidases in the molecular weight of the active form of the enzyme, the nature of its bound metal cofactors and its substrate specificity. Sephadex G-200 chromatography and polyacrylamide-gel electrophoresis both show that the catalytically active enzyme species has a mol.wt. of approx. 70000, which agrees reasonably well with the value found for the subunit molecular weight by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and analytical ultracentrifugation in the presence of 6M guanidinium chloride. Thus it may be concluded that the catalytically active form of the enzyme is a monomer of mol.wt. 70000–80000; this value is close to that reported by Baylin & Margolis (1975) for an amine oxidase from pregnancy plasma, which also would appear to be a monomer. The sedimentation-equilibrium ultracentrifugation results in the present study, which indicated a single species of mol.wt. 235000, may be explained on the basis of concentration-dependent aggregation, to dimeric and tetrameric species, which are in rapid equilibrium with each other (Williams, 1972). This explanation is consistent with kinetic evidence for aggregation in the enzyme (R. D. Waight & W. G. Bardsley, unpublished work).

The placental enzyme has 1 g-atom each of copper and manganese firmly bound per 70000 mol.wt. unit. The less than stoichiometric values for the copper and manganese content obtained from integration of the Q-band e.s.r. spectrum could be due to any of three reasons: first, the difficulty of maintaining exactly the same instrumental conditions for sample and standard; secondly, microwave-saturation effects, particularly with the manganese; thirdly, the possibility of spin-spin interactions between the copper and manganese centres. In common with other copper-containing amine oxidases, addition of the amine substrate does not reduce the Cu(II) centre. From Fig. 8, it appears that addition of CN^- abstracts copper from the enzyme and reduces it to the diamagnetic Cu(I) state; this explanation is consistent with the observed time-dependent inhibition of the enzyme by CN^- (M. J. C. Crabbe, unpublished work) and of pig kidney diamine oxidase by copper-chelating agents (Bardsley *et al.*, 1974a).

In view of the observed substrate specificity, the enzyme may act in the placenta to catalyse cross-linking of collagen precursors rather than solely in protecting the foeto-placental unit against elevated concentrations of biogenic amines as proposed pre-

vously (Bardsley *et al.*, 1974b). The human placental enzyme could then be at a focal control point in the metabolic activity of the foeto-placental unit during gestation, the regulation of its activity influencing, not only placental biogenic amine metabolism, but also placental collagen biosynthesis and possibly placental growth owing to alteration in polyamine concentrations influencing the rate of cell division.

Although many of the properties of this enzyme would place it close to a classical diamine oxidase, its metal cofactor requirements and novel substrate specificity make this a unique enzyme and only the third (after pyruvate carboxylase and manganese-containing superoxide dismutase) to contain tightly bound Mn^{2+} .

We thank Professor North for provision of laboratory space to M. J. C. C. in the Astbury Department of Biophysics, Leeds University, Mr. R. Boyes, Astbury Department of Biophysics, Leeds University, for the equilibrium analytical ultracentrifuge runs, Mr. M. Hart, Department of Chemistry, University of Manchester, for atomic-absorption analyses, Professor V. R. Tindall, Department of Obstetrics and Gynaecology, St. Mary's Hospital, Manchester, for support, the nursing staff at St. Mary's Hospital, Manchester, for assistance in obtaining placentae, the Medical Research Council for providing a Cary 118C spectrophotometer used in this study and Dr. F. Steven for providing special chemicals.

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