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Covalently bound pyrroloquinoline quinone is the organic prosthetic group in human placental lysyl oxidase

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Treatment of purified human placental lysyl oxidase with 2,4-dinitrophenylhydrazine (DNPH) resulted in a large spectral change and inhibition of enzyme activity. Proteolytic degradation of the derivatized enzyme yielded only one single coloured product, which was spectrally and chromatographically identical with the C-5 hydrazone of PQQ (pyrroloquinoline quinone) and DNPH. Since this represents the first example of a PQQ-containing enzyme in man, possible implications of the finding are discussed.

INTRODUCTION

Copper-containing amine oxidases (EC 1.4.3.6) catalyse the following reaction:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

The nature of the organic prosthetic group involved in this reaction has been a matter of debate for many years, the issue being whether or not it was pyridoxal phosphate (PLP) (see Yadav & Knowles [1] for a recent review and analysis of the available data). It now seems that this long-standing problem has been solved, since the cofactor in bovine serum amine oxidase [2] and porcine kidney diamine oxidase (R. A. van der Meer, unpublished work), appears to be covalently bound PQQ, a compound already shown to be the cofactor in a number of different bacterial oxidoreductases [3, 4].

Several human copper-containing amine oxidases have been found, so that, if they should also contain PQQ, it might imply that this novel compound has remained unrecognized as a vitamin in man. To shed light on this, a straightforward approach seemed first to elucidate the nature of the cofactor in a human amine oxidase. Since human placentas are the only tissue which can be obtained in quantities large enough to characterize such an enzyme and since purification of placental lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) has been achieved [5], this enzyme was chosen as the object of study.

MATERIALS AND METHODS

Purification of lysyl oxidase

The methods of Kuivaniemi *et al.* [5] and Kagan *et al.* [6] were adopted and modified for the isolation of the enzyme from human placental tissue. All procedures were carried out at 0–4 °C.

Six placentas (about 3.2 kg wet weight) were rinsed several times with 10 mM-Tris/HCl buffer, pH 7.8, containing 0.9% NaCl and 2 mM-EDTA. The tissue was passed through a meat grinder and subsequently suspended in 0.1 M-sodium phosphate, pH 7.0, containing 10 mM-EDTA (1 ml of buffer/g of ground tissue) and the mixture homogenized in a Sorvall Omnimixer. The homogenate was centrifuged at 13700 *g* for 20 min (9000 rev./min; Sorvall GS-3 rotor) and the pellet resuspended in the same buffer and centrifuged as described above. The pellet was then suspended (2 ml/g) in 50 mM-sodium phosphate, pH 7.8, containing 4.3 M-urea, the mixture stirred overnight and centrifuged at 13700 *g* for 30 min. To the supernatant, 1 litre of 50 mM-sodium phosphate, pH 7.8, and 400 ml of Cibacron Blue–Sepharose (equilibrated with the same buffer), prepared according to reference [7], were added. The mixture was agitated for 4 h and the affinity material removed by centrifugation. The pellet was suspended in the same buffer and the slurry packed into a column. The column (26 cm × 4.4 cm) was washed with 50 mM-sodium phosphate, pH 7.8, containing 1 M-NaCl, until an A_{280} value of 0.05 was reached, and the enzyme was eluted with 50 mM-sodium phosphate, pH 7.8, containing 6 M-urea, at a flow rate of 137 ml/h. The pooled enzyme fractions were dialysed against 0.2 M-sodium phosphate, pH 7.0, and the dialysis residue concentrated by pressure filtration.

Enzyme assay

Activities were determined at 25 °C by measuring the rate of O_2 consumption with a Clark oxygen electrode cell. The assay mixture (1.5 ml) contained 50 mM-sodium phosphate, pH 8.0, 3 mM-1,4-diaminopentane and enzyme in the appropriate concentration.

Protein determination

Protein concentrations were measured as described by Bradford [8], with bovine serum albumin as a standard.

Adduct isolation

Derivatization of the prosthetic group in the enzyme was achieved by incubating 50 ml of enzyme solution (17 mg of enzyme in 0.2 M-sodium phosphate, pH 7.0) with 10 μl of 0.1 M-DNPH in conc. H_3PO_4/ethanol (1:1, v/v) [9] at 40 °C for 16 h. During the incubation the mixture was oxygenated by blowing a stream of O_2 over the solution. Subsequently, the pH of the solution was adjusted to 7.5 with 1 M-NaOH and proteolysis performed by incubating with 1 mg of Pronase E (Boehringer) at 40 °C for 6 h. After proteolysis, the solution was adjusted to pH 2.0 with conc. HCl and passed through a Seppak C_{18} cartridge equilibrated with water (adjusted to pH 2.0

Abbreviations used: DNPH, 2,4-dinitrophenylhydrazine; PQQ, pyrroloquinoline quinone; PLP, pyridoxal phosphate.

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with HCl). After washing subsequently with 10 ml of water (pH 2.0), 10 ml of 10% methanol, and 100 ml of water, the red-coloured adduct was eluted with methanol.

Preparation of the model compound

The C-5 hydrazone of PQQ and DNPH was prepared by adding a slight excess of DNPH reagent {0.1 M-DNPH in conc. H_3PO_3/ethanol (1:1, v/v) [9]} to a saturated solution of PQQ in methanol at 40–50 °C. The suspension was stirred for 10 min at 50 °C, whereafter the copious precipitate was collected and washed with cold methanol. The orange–red solid was dissolved in large amounts of boiling methanol, affording the hydrazone as a microcrystalline orange solid upon cooling.

Analytical procedures

Reversed-phase h.p.l.c. was performed on a Waters h.p.l.c. system with an RCM 100 module containing a 10 μm C_{18} RCM cartridge. The eluent (flow rate 1.5 ml/min) consisted of a linear gradient (20 min) of 7–63% methanol in 10 mM-sodium phosphate, pH 7.0, containing 10 mM-NH_4Cl. The eluates were monitored with a Hewlett–Packard 1040 A photodiode-array detector, taking absorption spectra of the eluted peaks, upslope, at the top, and downslope, to check homogeneity and identity.

RESULTS

Enzyme purification

Solubilization of lysyl oxidase requires buffers with high concentrations of urea [10] and the enzyme has affinity for Cibacron Blue [11]. On the basis of these properties the present purification procedure was able to achieve a homogeneous enzyme, as revealed by electrophoresis in the presence of SDS, showing a main band of M_r 30000 and one weakly visible band (this band probably originates from a degradation product of the enzyme; see ref. [12]). The final preparation had a specific activity of 43615 units/mg of protein. Unfortunately this value cannot be compared with that reported by others [13], since quite different enzyme assays were used. However, all the following properties are in accordance with the view that the preparation contains human lysyl oxidase: the M_r of the subunit is identical with that found by others [13]; the absorbance spectrum (Fig. 1) resembles that of bovine aortic lysyl oxidase; besides 1,4-diaminopentane, the enzyme was also able to oxidize 1-aminobutane and ethanolamine, substrates that are not oxidized by other placental amine oxidases; enzyme activity was irreversibly blocked after incubation with 5 μM-β-aminopropionitrile, a concentration that is 100-fold lower than that required to block other amine oxidases [14] and is in accordance with the high sensitivity of the enzyme to this compound [15].

Enzyme derivatization

The procedure used is essentially similar to that originally described for bovine serum amine oxidase [2]. In the meantime, however, the method has been substantially improved, since it was found that quantitative yields of hydrazone could be obtained applying high O_2 concentrations during derivatization (R. A. van der Meer, unpublished work). By using this condition, it was found that the activity of lysyl oxidase completely disappeared and that the absorption spectrum changed into that of DNPH-derivatized bovine serum amine oxidase [2].

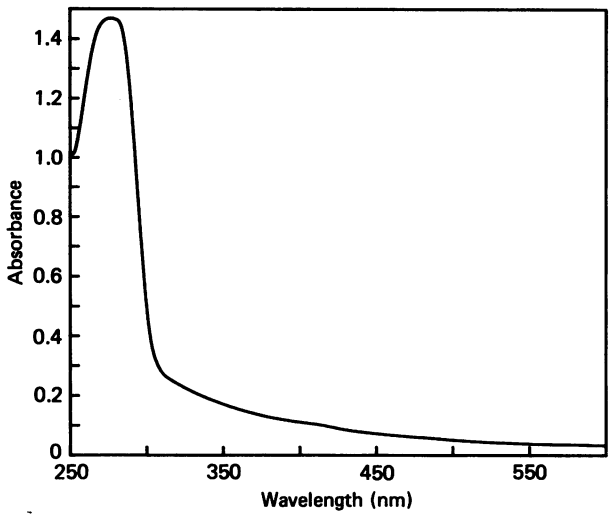

Fig. 1. Absorption spectrum of lysyl oxidase

The spectrum was taken of the final enzyme preparation in 0.2 M-sodium phosphate buffer, pH 7.0 (1.4 mg of protein/ml).

Adduct isolation and characterization

The present procedure developed to prepare the model compound is more convenient than that used previously [2] and yields a product that is homogeneous and spectrally and chromatographically (Fig. 2) identical with the C-5 hydrazone of PQQ and DNPH. Similarly, adduct isolation has been simplified, since it was found that detachment of the adduct from the protein chain is possible with only one proteolytic step, namely by the use of Pronase E (R. A. van der Meer, unpublished work). Applying this procedure to derivatized lysyl oxidase, only one single coloured compound was detected having the chromatographic and spectral properties of the model compound (Fig. 2). Assuming that one subunit contains one covalently bound PQQ molecule, the yield of adduct was calculated to be 40% (on the basis of a molar absorption coefficient of 31400 litre·mol^{-1}·cm^{-1} at 450 nm, as determined for the model compound). However, most copper-containing amine oxidases are dimers, and titration with hydrazines reveals only one carbonyl group per enzyme molecule [1]. Thus, if the same holds for lysyl oxidase, nearly quantitative isolation of the adduct was achieved.

DISCUSSION

Just like bovine serum amine oxidase [2] and porcine kidney diamine oxidase (R. A. van der Meer, unpublished work), human placental lysyl oxidase contains covalently bound PQQ. After proteolysis of the derivatized enzyme, the C-5 hydrazone of PQQ and DNPH was the only coloured product observed, most probably in quantitative yield (see the Results section). Thus it is highly improbable that the enzyme has also other carbonyl-group containing cofactors, such as for instance PLP. The absence of PLP is in accordance with the results of Williamson *et al.* [11], who failed to detect this compound in bovine aortic lysyl oxidase.

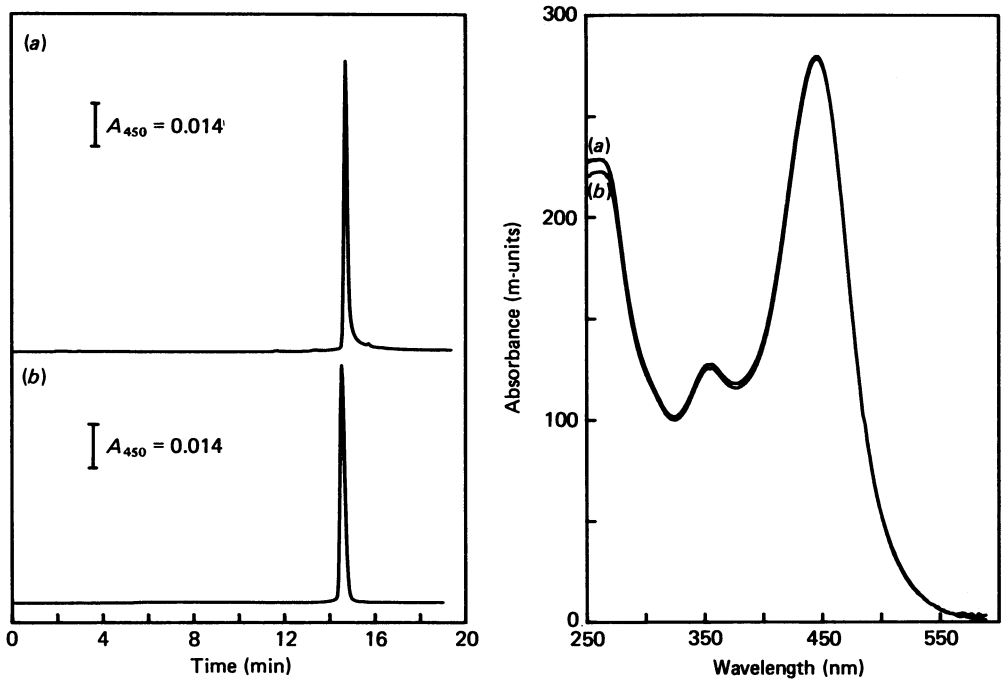

Fig. 2. Chromatograms (left) and absorption spectra (right) of the adduct (*a*) isolated from enzyme treated with DNPH and the model compound (*b*)

The Seppak eluates were injected on to a 10 μm C_{18} RCM cartridge in the h.p.l.c. system described in the Materials and methods section. The eluate was monitored at 450 nm. The absorption spectra were taken at the top of the peaks and they were normalized at their absorption maximum (446 nm).

A large number of disorders in human functioning have been related to the lowering of copper-containing-amine-oxidase activities [16]. It should be realized, however, that these studies were mostly performed on artificial model systems in which non-specific methods like depletion of copper or administration of carbonyl-group blocking agents were applied so that the relationships have not been unequivocally established. In this respect the discovery of PQQ as the organic prosthetic group could open new avenues as design of specific inhibitors for the enzymes or deprivation of PQQ by blocking its biosynthesis may now become possible. Conversely, the finding will also stimulate studies on the role of PQQ as a vitamin. If it appears that biosynthesis is absent, certain deficiences might be restored on administration of this compound.

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