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Crystallization of Expressed Porcine Kidney D-Amino Acid Oxidase and Preliminary X-Ray Crystallographic Characterization[1]

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The cDNA for porcine kidney D-amino acid oxidase (DAO) was cloned by means of the reverse transcription-polymerase chain reaction system from porcine kidney RNA and over-expressed in *Escherichia coli* which had been transformed with a vector containing the DAO cDNA. The expressed DAO was purified to homogeneity by a three-step procedure, *i.e.*, heat-treatment, DEAE Sepharose column chromatography, and hydroxyapatite column chromatography. The purified DAO preparation, rDAO (recombinant DAO), showed an identical UV-visible absorption spectrum and catalytic activity with those of the wild-type enzyme purified from porcine kidney. Crystallization of rDAO was performed by the hanging-drop method and crystals of suitable quality for X-ray crystallography were obtained. The crystals so obtained diffracted to 2.5 Å with a conventional X-ray source, and to 2.0 Å with synchrotron radiation. The crystals belong to the orthorhombic space group $P2_12_12_1$ with unit cell dimensions of $a=110.3$, $b=92.9$, $c=71.6$ Å. A V_m value of 2.35 Å^3/Da indicates that there are two subunits related by a twofold non-crystallographic axis in the asymmetric unit. Two heavy atom derivatives have been identified.

Key words: crystallization, D-amino acid oxidase, expression, flavoenzyme, X-ray crystallography.

D-Amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] (DAO) was discovered more than 60 years ago as the first mammalian flavoenzyme (*1, 2*) and was the first mammalian flavoenzyme to be crystallized (*3, 4*). DAO is one of the most extensively studied flavoenzymes from physiological, enzymological, physicochemical, and thermodynamic points of view (*5*). Despite the broad range of investigation, the physiological significance of DAO in conjunction with the controversial and enigmatic roles of D-amino acids in the biological kingdom remains cryptic. Moreover, the catalytic mechanism of DAO is still a matter of controversy; the reaction has been proposed to proceed *via* a discrete carbanion intermediate generated by abstraction of the substrate's α-proton followed by covalent linkage of the intermediate to the flavin ring (*6, 5* and references cited therein) or *via* the α-proton abstraction in concert with the transfer of the electron pair from the amino nitrogen to flavin (*7, 8*).

DAO possesses one molecule of FAD noncovalently bound to each subunit of molecular mass 39 kDa (*9, 10*). The primary structure (*9*) and nucleotide sequence of the cDNA (*11*) are known. Numerous chemical modification studies as well as mutagenesis studies have provided clues to identify catalytically important (or unimportant) residues (*5* and references cited therein). However, neither the primary structural information nor the data from modification/mutation studies suffice to clarify the catalytic events of DAO at the molecular or submolecular level. There is no doubt whatsoever that the three-dimensional structure of this enzyme would provide fundamental insight into the catalytic mechanism of DAO. A preliminary X-ray crystallographic study was published by Bolognesi *et al.* (*12*), but the complete structural analysis has yet to be presented. Based on our conviction that the heterogeneity of DAO in kidney cortex (*13, 14*) has hampered the growth of crystallographically adequate crystals, we developed an expression system for homogeneous DAO using a single cDNA introduced into *Escherichia coli*. We report herein the expression system for DAO from DAO cDNA from porcine kidney, purification of the expressed DAO (rDAO), its crystallization and the preliminary X-ray characterization.

MATERIALS AND METHODS

pET-11d, an expression vector, was purchased from Novagen, M-MLV reverse transcriptase from GIBCO BRL, and restriction endonucleases and other DNA processing enzymes from Takara Shuzo. Hen egg lysozyme, isopropyl-1-thio-β-D-galactopyranoside (IPTG), FAD, DEAE Sepharose CL6B, and polyethylene glycol 4000 were from

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Abbreviations: DAO, D-amino acid oxidase; rDAO, DAO purified from *Escherichia coli* with recombinant DNA; EMTS, ethylmercurithiosalicyclic acid, sodium salt; IPTG, isopropyl-1-thio-β-D-galactopyranoside; RT-PCR, reverse transcription-polymerase chain reaction.

Sigma, Wako Pure Chemical Industries, Sigma, Pharmacia, and Merck, respectively. The molecular mass standards used in SDS-PAGE were from Bio-Rad. Other chemicals were of the highest grade available and were used as supplied.

Construction of the expression plasmid for DAO was performed as follows. Using total RNA extracted from porcine kidney as described (*15*), cDNA was prepared with reverse transcriptase and oligo(dT) as a primer. The DAO cDNA was isolated by polymerase chain reaction with a Perkin Elmer thermal cycler PJ2000 from porcine kidney cDNA using two primers, 5′-CGTGTGGTGGTGATTGGAGCA-3′ and 5′-GATGTCTTCAGAGGTGGGATG-3′, which were synthesized based on the DAO cDNA reported by Fukui *et al.* (*11*). These primers were to amplify a 1,048 bp fragment containing the region between the nucleotide immediately downstream from the AUG start site and the seventh nucleotide downstream from the TGA termination codon. After 30 cycles of denaturation (1 min, 94°C), annealing (1 min, 67°C), and elongation (30 s, 72°C), the PCR product of the expected size was cut from the 1% agarose gel and purified. The resulting DAO fragment was phosphorylated with T4 polynucleotide kinase, and then inserted into the expression vector pET-11d which was linearized at a unique *Nco*I site and treated with the Klenow fragment. Finally, the expression plasmid pET-DAO was obtained. The amplified DAO cDNA was sequenced with specific primers to confirm its authenticity.

Expression and purification of the recombinant DAO (rDAO) were done as follows. *Escherichia coli* BL21 (DE3) cells harboring pETDAO were grown in 126 liters of LB medium containing 50 μg/ml carbenicillin at 37°C to a turbidity (absorbance at 600 nm) of 0.6. IPTG was added to a final concentration of 0.01 mM and the cells were grown for a further 20 h. The cells were harvested by centrifugation and stored frozen at −20°C until use. The cell pellets were suspended in 17 mM sodium pyrophosphate, pH 8.3, containing 1 mg/ml sodium benzoate and 300 μM FAD and treated with 1 mg/ml lysozyme for 1 h in an ice bath. The cells were then disrupted by sonication and the cell debris was removed by centrifugation. The supernatant was heat-treated at 59°C for 3 min and rapidly cooled to below 10°C in an ice-water bath. After centrifugation, ammonium sulfate (144 g/liter) was added to the supernatant and the precipitate was collected by centrifugation and dialyzed extensively against 10 mM Tris hydrochloride, pH 8.0, containing 125 mM KCl and 200 μM sodium benzoate. The protein solution was applied to a DEAE Sepharose CL 6B column (3.2×25 cm) which had been equilibrated with the same buffer. The column was eluted with the same buffer and the yellow fractions were pooled and ammonium sulfate (200 g/liter) was added. The protein precipitate was collected by centrifugation and thoroughly dialyzed against 50 mM sodium phosphate, pH 6.8, containing 200 μM sodium benzoate. The dialyzed preparation was applied to a hydroxyapatite column (3.2×25 cm) equilibrated with the same buffer. The protein(s) was eluted with the same buffer. The yellow fractions, which contained a homogeneous protein with the molecular mass corresponding to DAO from porcine kidney as judged by SDS-PAGE, were collected and precipitated with 200 mg/ml ammonium sulfate. The enzyme stored at 0°C in the form of precipitate taken up in a minimum volume of the same buffer was stable for several months. The enzyme was purified as the complex with benzoate and was freed of benzoate by the procedure reported (*16*) when necessary.

The activity of rDAO was determined as the oxygen consumption at 25°C in a closed 1.5-ml reaction vessel equipped with a Galvani-type oxygen electrode (Sensonix Japan). The concentration of DAO was based on the molar extinction coefficient of 11,300 $M^{-1} \cdot cm^{-1}$ at 455 nm (*17*).

The amino acid and cDNA sequences were determined with an Applied Biosystems gas-phase protein sequencer and an Applied Biosystems 373A DNA sequencer, respectively.

Crystals of sufficient quality for X-ray crystallography were grown by vapor diffusion using the hanging drop method. For X-ray characterization, the yellow crystals of rDAO were mounted in glass capillaries with the mother liquor. Crystallographic analysis of these crystals was performed with an Enraf-Nonius precession camera. Data collection was performed at 20°C on a Rigaku RAXIS IIc imaging plate detector with graphite-monochromated CuKα X-rays produced from a Rigaku RU-H2R rotating anode X-ray generator operating at 40 kV, 100 mA, and on a screenless Weissenberg camera (*18*), using synchrotron radiation with a wavelength of 1.00 Å at beam line BL6A of the Photon Factory (KEK, Japan).

RESULTS AND DISCUSSION

Expression of DAO in E. coli and Purification of rDAO—In order to obtain a homogeneous DAO preparation suitable for X-ray crystallography, we developed an expression system of porcine kidney DAO as described under "MATERIALS AND METHODS." To confirm the authenticity of the expression system we sequenced the expression plasmid and found that the sequence encoding DAO was indeed identical with that of the DAO cDNA reported by Fukui *et al.* (*11*) (results not shown). Pollegioni *et al.* have recently expressed DAO in *E. coli* (*19*) in a slightly different system from the one employed in this study; we have utilized a different expression vector, a different strain of *E. coli* and a different IPTG concentration for induction. The system developed in the present study turned out to be highly efficient. Judging from the SDS-PAGE profile of the cell extract, the expressed DAO amounted to about 40% of the total protein (Fig. 1).

The expressed protein was purified according to the procedure described under "MATERIALS AND METHODS" and the preparation thus obtained was homogeneous as judged by SDS-PAGE, which showed a single protein band corresponding to the molecular mass of 39 kDa consistent with porcine kidney DAO (Fig. 1). Furthermore, the N-terminal amino acid sequence of the purified protein was identical to that of native DAO up to residue 20 (data not shown). The absorption spectrum of the preparation was identical to that of porcine kidney DAO (Fig. 2). Moreover, the enzymatic properties of the purified preparation coincide with those of native DAO; K_m and k_{cat} values obtained from kinetic studies with D-alanine as the substrate and 0.268 μM enzyme were 1.82 mM and 478 min^{-1}, respectively. Thus, the expressed protein is a D-amino acid oxidase not very different from porcine kidney DAO; we still lack direct proof that the expressed DAO is 100% identical to DAO from pig kidney. We therefore designate

this expressed protein rDAO, recombinant DAO, to distinguish it from DAO purified from porcine kidney. Using this purification procedure, about 2 g of rDAO was obtained from 126 liters of the IPTG-induced culture.

Crystallization of rDAO—Drops of the rDAO solution (3.5 mg/ml) initially in 120 mM sodium acetate, 60 mM sodium citrate, pH 6.3, 200 μM sodium benzoate, and 5% polyethylene glycol 4000 were equilibrated at 20°C against a reservoir containing 200 mM sodium acetate, 100 mM sodium citrate, pH 6.3, and 30% polyethylene glycol 4000. After a few days, small yellow prisms started to grow and were used as seeds for growing large crystals under the same hanging drop conditions. Within 2 weeks, crystals grew to dimensions of about 0.2×0.3×0.8 mm (Fig. 3). Since benzoate (initially 200 μM) was present in the drop, the crystalline rDAO thus obtained is expected to be the complex form with benzoate.

X-Ray Crystallographic Characterization of rDAO—The space group of the rDAO crystal has been determined by precession photography as $P2_12_12_1$ with the cell dimensions of $a=110.3$, $b=92.9$, $c=71.6$ Å. The assumption of a single dimer (78 kDa) in the asymmetric unit gives a V_m value of 2.35 Å^3/Da, which is within the range of commonly found values (*20*). A set of native data was collected to 2.5 Å resolution on a Rigaku RAXIS IIc using 2.5° oscillation over a range of 87.5° (96.5% complete with 23,155 unique reflections) and the R_{merge} value based on the intensity data was 8.3% (Fig. 4). The data sets for the crystals soaked in 1 mM *m*-iodobenzoate and 1 mM sodium ethylmercurithiosalicylate (EMTS) were also collected to 2.5-Å (R_{merge} = 6.1) and 3.0-Å (R_{merge} = 9.3%) resolution, respectively, on the same system used for the native crystal. The native data up to 2.0-Å resolution were also collected with a screenless Weissenberg camera, using synchrotron radiation. The self-rotation function (*21*) computed with the native data indicates a non-crystallographic twofold axis

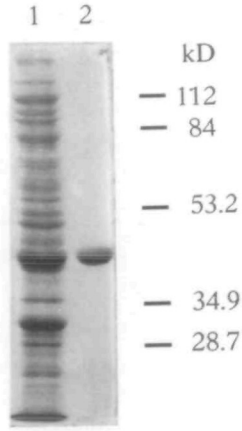

Fig. 1. **The SDS-PAGE patterns of the cell extract (lane 1) and rDAO (lane 2).** The positions of the molecular mass standards are shown to the right (values as indicated).

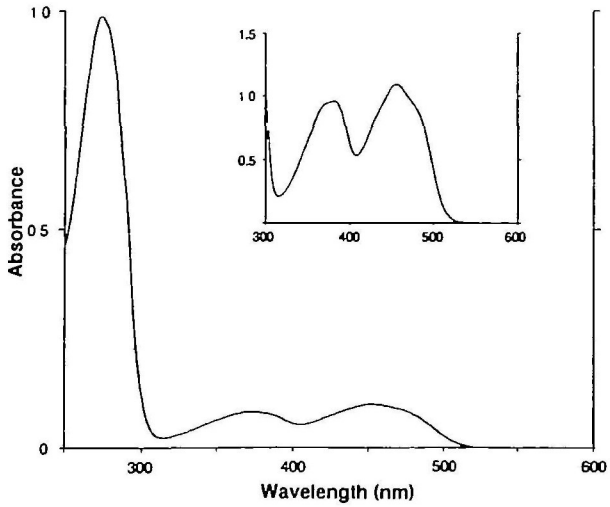

Fig 2 **The absorption spectrum of rDAO.** The purified rDAO was freed of benzoate as described under "MATERIALS AND METHODS." The concentrations of rDAO were 8 8 and 96.5 μM (inset).

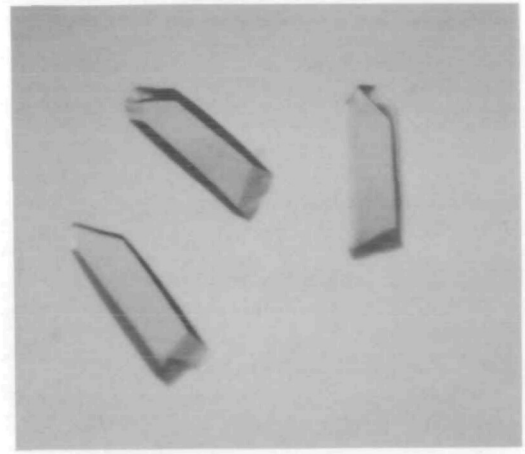

Fig. 3 **Photograph of crystals of rDAO grown by the hanging-drop vapor diffusion method.**

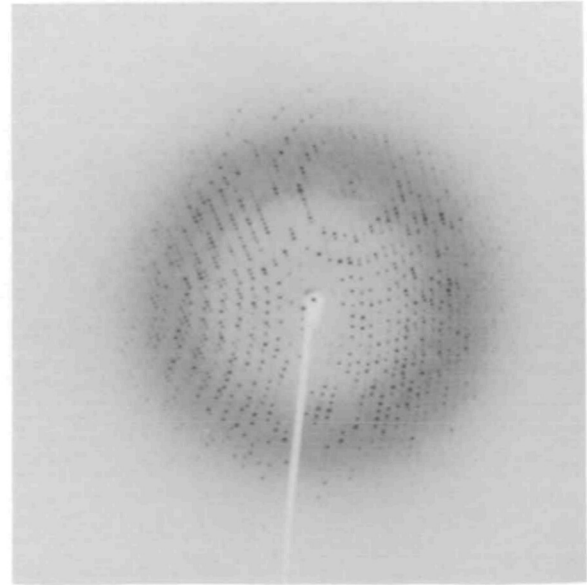

Fig. 4. **Oscillation photograph of a native crystal of porcine kidney rDAO taken with CuKα radiation on a Rigaku RAXIS IIc imaging plate detector (2.5° oscillation).**

consistent with the presence of one dimer in the asymmetric unit. Two iodine sites of the *m*-iodobenzoate derivative were located from the difference Patterson map and seven mercury sites of the EMTS derivative were located from the isomorphous difference Fouriers using the iodine-derived phases (*21*). We are now in the process of refining the heavy atom parameters for the two derivatives and searching for more heavy atom derivatives for determination of the high-resolution three-dimensional structure of rDAO.

It should be emphasized that the crystal form of rDAO obtained herein is quite different from that of Bolognesi *et al.* (*12*) for DAO from porcine kidney in many respects, *i.e.*, the space group, the cell dimensions, the V_m value and the number of subunits in the asymmetric unit. We do not know currently the reason for these differences. They may have stemmed from the difference in the crystallization conditions or the difference in the protein source; our protein was rDAO, whereas theirs was native DAO purified from porcine kidney. Bolognesi *et al.* employed a batch method for crystallization with 10 mM borate buffer, pH 8.3, containing 0.7 M ammonium sulfate and 0.2 mM sodium benzoate (*12*), whereas we employed a vapor diffusion method using a different buffer, precipitant and salt at a different pH.

One other point to be stressed is that the asymmetric unit contains the dimer of rDAO. It has been demonstrated by Tojo *et al.* (*22*) that DAO holoenzyme exhibits concentration-dependent polymerization behavior and that the basic unit for the polymerization is the dimer. Our result showing the dimeric unit in the crystal is consistent with their polymerization model and with the negative value for the second virial coefficient (*22*), which usually represents weak intermolecular, *i.e.*, interdimer in this case, attractive forces. With regard to this dimeric unit in the crystal, we emphasize here that DAO is catalytically active either in its monomeric or dimeric form and that the catalytic constants for monomeric and dimeric forms are different from each other (*23, 24*). In clear contrast to this dimeric unit in our crystals, the number of subunits present in the asymmetric unit in the crystals reported by Bolognesi *et al.* was in the range from 8 to 16 (*12*). This difference may be due to the difference in the crystallization conditions or in the protein source as described above.

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