In Vivo and in Vitro Expression of Porcine D-Amino Acid Oxidase: In Vitro System for the Synthesis of a Functional Enzyme†

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ABSTRACT: In vivo expression of D-amino acid oxidase (EC 1.4.3.3, DAO), one of the principal and characteristic enzymes of the peroxisomes of porcine kidney, was examined by use of cloned complementary DNA [Fukui, K., Watanabe, F., Shibata, T., & Miyake, Y. (1987) *Biochemistry 26*, 3612–3618]. RNA blot hybridization analysis revealed that DAO is expressed abundantly in kidney and liver, is expressed significantly in brain, but is not expressed in lung of pig. Three mRNA species were expressed in kidney and liver, but only one was detected in brain. These results show the presence of tissue-specific regulation of DAO gene expression. In vitro expression of a functional enzyme was achieved through the construction of a recombinant plasmid containing an SP6 promoter and a restriction enzyme fragment of cDNA to generate a DAO-specific RNA transcript. The in vitro translation product of the capped RNA transcript showed significant catalytic activity, which was inhibited strongly by benzoate, a potent inhibitor of DAO. The kinetic properties of the in vitro synthesized enzyme were comparable to those of the purified enzyme from porcine kidney. It is now possible to synthesize a functional D-amino acid oxidase in vitro and to investigate its structure–function relationships.

D-Amino acid oxidase [D-amino acid:O_2 oxidoreductase, EC 1.4.3.3] (DAO)[1] is one of the flavoproteins with flavin adenine dinucleotide (FAD) as the prosthetic group that catalyzes the oxidative deamination of D-amino acids. Systematic studies on DAO activity in various tissues revealed its existence in kidney proximal tubules, liver (Krebs, 1935), and certain parts of the brain (Dunn & Perkoff, 1963). This enzyme was reported to be intracellularly localized in peroxisomes, and DAO is regarded as a characteristic marker enzyme of the peroxisomes in porcine kidney (de Duve & Baudhuin, 1966). Extensive studies have been carried out to clarify the physicochemical properties and reaction mechanism of this enzyme. Although several clues as to the function of the enzyme have been reported (Hamilton et al., 1979; Hamilton & Buckthal, 1982), D-amino acids do not appear to be intermediates in normal mammalian metabolism, and the biological significance of this enzyme remains to be elucidated.

One of the approaches taken for investigating the function of this enzyme was to use an enzyme inhibitor in an in vivo study. D-Propargylglycine (D-2-amino-4-pentynoic acid, D-PG), an acetylenic substrate for DAO, was oxidatively deaminated by DAO, with accompanying inactivation of the enzyme (Horiike et al., 1975; Marcotte & Walsh, 1978a,b). The inactivation reaction was demonstrated to be due to a dynamic affinity labeling with D-PG, which caused irreversible inactivation of the enzyme through covalent modification. Intraperitoneal injection of D-PG into rats resulted in massive glucosuria and amino aciduria (Nakajima et al., 1981). These observations indicated a dysfunction of the renal proximal tubules, since they contain a high level of DAO (Chan et al., 1979) and transport glucose and amino acids in the nephrons.

We have demonstrated the active biosynthesis of this enzyme in a functionally differentiated cell line, LLC-PK_1, derived from porcine kidney proximal tubules, and also in vitro DAO synthesis using pig kidney mRNA and free or membrane-bound polysomes. The intracellular site of synthesis of DAO was concluded to be free polyribosomes, as is the case for other peroxisomal proteins (Fukui et al., 1986). Recently, we isolated cDNA clones from a porcine kidney cDNA library and determined the complete nucleotide sequence of the cDNA. The primary structure of DAO, predicted from the nucleotide sequence, does not contain a cleavable presequence in its amino-terminal region for its translocation to peroxisomes, indicating the presence of a recognizable signal for translocation within the polypeptide. Analysis of the expression of the DAO gene in porcine kidney by RNA blot hybridization showed that three mRNA species were produced through the use of multiple polyadenylation signal sequences (Fukui et al., 1987).

In this study, the in vivo synthesis of this enzyme was first investigated by Northern blot hybridization to determine the tissue specificity of DAO gene expression, in search of some clues as to the physiological function of DAO. Since developments in molecular biology have made it possible to address problems concerning protein structure and function through the modification of cDNAs and the expression of mutant proteins, we used this approach for a study on DAO. First we tried to synthesize a functional enzyme in vitro using cloned cDNA. The biochemical, immunological, and enzymological properties of the in vitro synthesized enzyme obtained were compared with those of the authentic enzyme purified from porcine kidney.

Experimental Procedures

Materials. The reagents were obtained from the following sources: [α-^{32}P]dCTP (sp act. 410 Ci/mmol), D-[U-^{14}C]-alanine (sp act. 40 mCi/mmol), [^{35}S]methionine (sp act. 1145

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[1] Abbreviations: DAO, D-amino acid oxidase; kb, kilobase(s); FAD, flavin adenine dinucleotide; SDS, sodium dodecyl sulfate; LDH, lactate dehydrogenase.

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Ci/mmol), ^{125}I-Protein A (sp act. 30 mCi/mg), and a rabbit reticulocyte lysate from Amersham, U.K.; *Escherichia coli* DNA polymerase I and restriction endonucleases from Toyobo Co., Japan; nitrocellulose filters from Advantec, Japan, and Schleicher & Schuell, West Germany; agarose type I from Sigma; guanidinium thiocyanate from Fluka, Switzerland; pSP64 DNA, SP6 RNA polymerase, and RNasin from Promega-Biotec; and guanylyltransferase from Bethesda Research Laboratories. All other chemicals used were of analytical grade and purchased from Sigma, Nakarai Chemicals, Japan, and Wako Pure Chemical Co., Japan.

RNA Preparation. Total RNAs were extracted from porcine kidney, lung, liver, and whole brain with guanidinium thiocyanate (Chirgwin et al., 1979), and poly(A+) RNAs were isolated by subjecting the total RNAs to oligo(dT)–cellulose column chromatography (Aviv & Leder, 1972).

Northern Blot Hybridization. Poly(A+) RNA samples (5 μg per lane) were denatured at 50 °C in a buffer containing 1 M glyoxal, 50% (v/v) dimethyl sulfoxide, and 10 mM NaH_2PO_4/Na_2HPO_4, pH 6.5–7.0, and then separated by 1.5% agarose gel electrophoresis in 10 mM phosphate buffer, pH 6.5–7.0. The RNAs were then transferred to a nitrocellulose filter by the procedure of Thomas (1980). After overnight transfer in 20× SSC, the blots were baked at 80 °C for 2 h. The filter was hybridized with a DNA fragment derived from a cDNA insert that was labeled by nick translation with [α-^{32}P]dCTP to obtain a specific activity of 150–300 cpm/pg (Rigby et al., 1977). The hybridization and filter washing conditions were as described (Honjo et al., 1979).

Construction of a Plasmid. The construction of a plasmid containing the coding sequence for porcine kidney DAO was performed as follows. A *Sal*I–*Pvu*II fragment of plasmid pDAO-10 (Fukui et al., 1987) that contains the coding sequence was inserted into the *Sal*I–*Sma*I sites of the pSP64 vector (Melton et al., 1984; Krieg & Melton, 1984). The resulting plasmid contained the DAO gene in the correct orientation for transcription from the SP6 phage promoter.

Transcription with the SP6 Phage RNA Polymerase. The plasmid (pSP64-DAO) was linearized by *Eco*RI digestion and then transcribed in 100-μL aliquots of a solution containing 40 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each ATP, CTP, UTP, and GTP, 10 mM dithiothreitol, 100 units of RNasin, 5 μg of linearized plasmid, and 20 units of SP6 RNA polymerase. The reactions were performed at 37 °C for 4 h. After RNA synthesis, the DNA template was digested by incubation with 5 units of DNase at 37 °C for 15 min. The in vitro synthesized RNAs were extracted with phenol–chloroform and then recovered by ethanol precipitation. The 5′-terminal cap structures were transferred to the synthesized RNA transcripts with guanylyltransferase. Five micrograms of the synthesized RNAs was incubated at 37 °C for 45 min with 5 units of guanylyltransferase in a 150-μL reaction mixture containing 50 mM Tris-HCl, pH 8.0, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 1 unit/μL RNasin, 0.1 mM *S*-adenosylmethionine, and 40 μM GTP.

In Vitro Translation. Translation was performed at 30 °C for 2 h as reported (Fukui et al., 1986). Five micrograms of the capped RNA transcripts was translated in a 25-μL reaction mixture containing 20 μL of rabbit reticulocyte lysate, 10 μM each of amino acid, 50 μM FAD, and 20 units of RNasin. For apoenzyme synthesis, FAD was omitted. After incubation, the reaction mixture was diluted with 0.05 M sodium pyrophosphate buffer, pH 8.3, for the enzyme assay and SDS–polyacrylamide gel electrophoresis. Fluorography was performed for the products translated in the presence of 10 μCi of [^{35}S]methionine.

Enzyme Preparation. Porcine kidney D-amino acid oxidase was purified essentially by the procedure of Kubo et al. (1960). This preparation was treated with hydroxylapatite by the method of Massey et al. (1961) and then subjected to DEAE-Sephadex (A-50) column chromatography for final purification according to Curti et al. (1973). As benzoate was used to stabilize DAO during the purification and was bound to the purified enzyme, it was removed by passing the D-alanine-reduced enzyme through a Sephadex G-50 column equilibrated with 50 mM sodium pyrophosphate buffer (pH 8.3).

Enzyme Assay. Enzyme activity was determined in two ways. First, the amount of ^{14}C-labeled pyruvate produced from D-[^{14}C]alanine was measured directly. DAO purified from porcine kidney or the in vitro translation product, in 50 mM sodium pyrophosphate buffer (pH 8.3), was incubated with D-[^{14}C]alanine (0.5 μCi) in the presence of 30 μM FAD and 0.2 mM D-alanine for 90 min at 25 °C. After incubation, the reaction mixtures were fractionated by ion-exchange (TSK-Gel DEAE-2SW) high-performance liquid chromatography, and then, radioactivity in the pyruvate fraction was determined by simultaneous scintillation counting with a radioactive flow detector (Flo-One/Beta, Model IC, Radiomatic Instruments & Chemical). Second, the production of pyruvate was measured by the rate of decrease in absorbance at 340 nm of NADH in a coupled system containing lactate dehydrogenase. The assay mixture contained 20 μL of in vitro translation product, 50 μM FAD, 5.9 units/mL lactate dehydrogenase, 0.2% Triton X-100, 50 μM NADH, 20 mM D-alanine, and 50 mM sodium pyrophosphate buffer (pH 8.3), in a total volume of 500 μL. The reaction temperature was 25 °C. Since the reticulocyte lysate of the in vitro translation mixture contained sufficient amounts of catalase, the assay was performed without an added catalase. The molar extinction coefficient of NADH at 340 nm was taken as 6.3×10^3 M^{-1} cm^{-1}. The concentration of FAD was determined with a molar extinction coefficient of 11.3×10^3 M^{-1} cm^{-1} at 450 nm (Whitby, 1953). Enzyme concentrations of the order of 10^{-8}–10^{-9} M were employed for measurements of enzymatic activity. Spectrophotometric measurements were performed with a Hitachi spectrophotometer, Model 220.

Western Blotting. Western blotting was carried out as described (Burnette, 1981) for immunological detection and quantitative analysis of translation products. An aliquot (40%) of each in vitro translation mixture was subjected to SDS–polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically from the SDS–polyacrylamide gel to a nitrocellulose filter and then incubated with polyclonal anti-porcine DAO antiserum at 1000-fold dilution. The immune complexes were radiographically visualized through the binding of ^{125}I-protein A. The relative abundance of DAO was determined by quantitative scanning densitometry of autoradiographs with an LKB Ultrascan XL laser densitometer and integrator. This method was employed when signals were in the linear range of film sensitivity.

Results

Tissue-Specific Expression of DAO mRNA in Porcine Tissues. We reported previously the presence of three DAO mRNA species in porcine kidney (Fukui et al., 1987). To further study the in vivo expression of the DAO gene and to obtain a clue concerning the physiological role of DAO, Northern blot hybridization experiments were carried out with cloned cDNA as a hybridization probe (Figure 1).

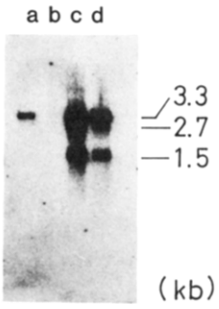

FIGURE 1: Tissue-specific expression of DAO mRNA in porcine tissues. Five micrograms of poly(A+) RNA per lane was fractionated by electrophoresis on a 0.7% agarose gel and then transferred to a nitrocellulose filter. The blots were hybridized with a nick-translated cDNA fragment (*Sal*I–*Pvu*II fragment of pDAO-10, 1.34 kb). The origins of poly(A+) RNA were as follows: (a) brain; (b) lung; (c) kidney; (d) liver.

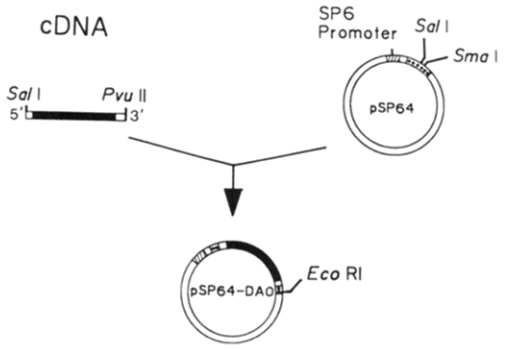

FIGURE 2: Schematic diagram of the construction of a recombinant vector for in vitro synthesis of DAO. A *Sal*I–*Pvu*II fragment comprising the whole coding region of the full-length porcine kidney D-amino acid oxidase cDNA (pDAO-10) was cloned into the *Sal*I and *Sma*I sites of pSP64. This vector uses an SP6 promoter for expression of the recombinant gene. The solid box represents the coding region for DAO; the open box in the cDNA fragment represents the untranslated region; the dotted column represents the polylinker region; the hatched column represents the SP6 promoter.

The cDNA fragment containing the protein-coding region of DAO detected three bands, whose mobilities corresponded approximately to 3.3, 2.7, and 1.5 kb, for porcine liver and kidney (lanes c and d). The hybridization signals of the 3.3- and 1.5-kb mRNAs were significantly more intense than that of 2.7-kb mRNA in both kidney and liver, suggesting different efficiencies of expression. Although equal amounts of poly(A+) RNAs were applied to the gel, the relative intensities of the three bands were lower in liver than in kidney.

In lung, no expression of the DAO gene was observed within the sensitivity range of the analysis. In brain, only the 3.3-kb mRNA was clearly detected, although the copy number was small.

Construction of a Recombinant Plasmid for in Vitro Synthesis of DAO. We attempted, as the next step, to synthesize DAO in vitro using an SP6 transcription system (Melton et al., 1984; Krieg & Melton, 1984) as an in vitro model system to prepare large amounts of the RNA transcript specific for porcine kidney DAO. The cDNA fragment of pDAO-10 (*Sal*I–*Pvu*II, 1.34 kb) containing the entire coding region for porcine kidney DAO was subcloned into a multicloning site, downstream of an SP6 promoter, of the pSP64 plasmid vector, which allowed the efficient synthesis of an SP6 promoter-directed RNA transcript for DAO. The resultant recombinant plasmid, designated as pSP64-DAO, is shown schematically in Figure 2. It contains the entire coding region for DAO plus 68 bp of the 5′ and 237 bp of the 3′ noncoding sequences, respectively.

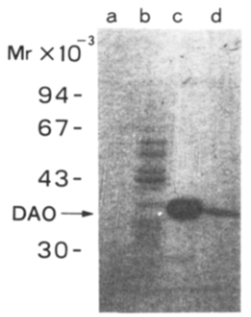

FIGURE 3: Fluorography of the in vitro translation product of pSP64-DAO following SDS–polyacrylamide gel electrophoresis. After incubation for in vitro translation, an aliquot (40%) of the reaction mixture was subjected to SDS–polyacrylamide gel electrophoresis and then analyzed by fluorography: (a) RNA (–); (b) total mRNA of porcine kidney (0.3 μg); (c) in vitro synthesized transcript capped with guanylyltransferase (0.2 μg); (d) in vitro synthesized transcript without capping (0.3 μg).

When this plasmid was linearized with *Eco*RI and then transcribed with SP6 RNA polymerase, a discrete 1.5-kb RNA was produced (data not shown). Northern blotting demonstrated hybridization of the RNA with the cDNA fragment used for plasmid construction (data not shown).

In Vitro Translation of a DAO-Specific RNA Transcript. To obtain the in vitro synthesized enzyme, RNA transcripts produced by the SP6 system were first capped with guanylyltransferase and then used as the template for translation in a rabbit reticulocyte lysate. The molecular properties of the in vitro translation product were first analyzed by SDS–polyacrylamide gel electrophoresis and fluorography using the product labeled with [^{35}S]methionine. Aliquots of the translation mixture were analyzed without any prior purification. As shown in Figure 3, a single discrete protein band was detected without any contaminating or smaller polypeptides resulting from premature termination of translation (lane c). The mobility of this protein band corresponded quite well with that of the purified enzyme (M_r = 38K). Capping of the RNA transcript increased the efficiency of in vitro translation, 18-fold, as judged by densitometric measurement (lanes c and d).

Since DAO has FAD as a prosthetic group, the amount of FAD in the translation mixture was determined with the FAD-depleted DAO apoenzyme. In this assay, FAD was not detected in the rabbit reticulocyte lysate (at most, less than 50 pM). Therefore, it is possible to synthesize the apoenzyme in vitro by simply omitting FAD from the reaction mixture. The presence (50 μM) or absence of FAD did not affect the amount of protein synthesis in vitro (data not shown).

The results indicated that the in vitro synthesized transcript directed the synthesis of a single protein in vitro with the same molecular weight as DAO.

Immunological Identification and Quantitation of the Translation Product. To characterize the translation product further, its immunoreactivity was analyzed with rabbit anti-porcine kidney DAO antiserum by Western blotting. As shown in Figure 4A, the capped RNA transcript directed the synthesis of a protein which showed positive reactivity with the antibody (lane a). No cross-reacting protein was detected in the reticulocyte lysate without an RNA transcript (lane b). The intensity of the band corresponding to authentic DAO (lane c) increased with the amount of DAO applied on the gel in a linear manner. When autoradiographic signals were within the linear range of film sensitivity, the amount of the in vitro synthesized immunoreactive translation product was determined by quantitative scanning densitometry (Figure 4B).

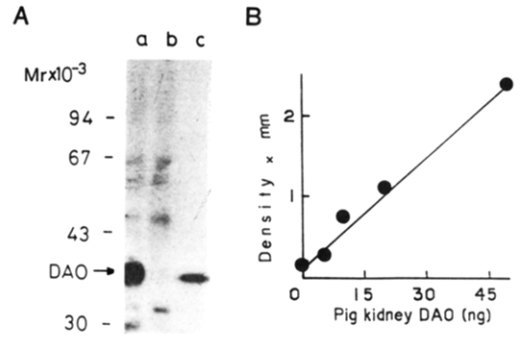

FIGURE 4: Western blotting analysis of the in vitro translation product. (A) (a) In vitro translation product (10% of the reaction mixture); (b) in vitro translation mixture without RNA; (c) authentic DAO purified from porcine kidney (10 ng). (B) Quantitation of DAO by Western blotting. Each filled circle represents the amount of authentic DAO applied to the gel and the relative intensity of the band on Western blotting as measured by densitometry.

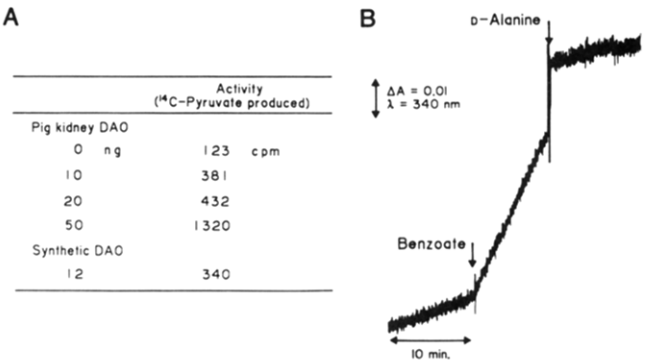

FIGURE 5: Catalytic activities of the in vitro synthesized DAO. (A) D-Alanine oxidation activity of the product. Authentic DAO and the in vitro synthesized DAO (12 ng of DAO protein, as judged by densitometric measurement) were incubated with D-[^{14}C]alanine in the presence of 30 μM FAD for 90 min at 25 °C. After incubation, the reaction mixtures were chromatographically fractionated, and then, the radioactivity in the pyruvate fraction was determined. (B) Spectrophotometric measurement of DAO activity and inhibition by benzoate. The time course of D-alanine oxidation activity was followed by means of an LDH-coupled assay (340-nm absorbance) as described under Experimental Procedures. The spectrophotometer trace obtained is presented without correction, the passage of time during the reaction being from right to left. D-Alanine was added at the point indicated by the first arrow, and sodium benzoate (1 mM) was added at the point indicated by the second arrow. The experiments were conducted at pH 8.3 and 25 °C.

With this procedure, about 250 ng of DAO protein was found to be produced in vitro from 5 μg of the capped RNA transcript.

Enzymatic Activities of the in Vitro Synthesized DAO and Inhibition by Sodium Benzoate. The catalytic activity of the translation product was examined in two ways. First, the oxidation of ^{14}C-labeled D-alanine was investigated. The in vitro translation mixture was incubated, in the presence of 30 μM FAD, with D-[^{14}C]alanine for 90 min at 25 °C. After incubation, the reaction mixture was fractionated by ion-exchange high-performance liquid chromatography, and then radioactivity in the pyruvate fraction was determined by simultaneous scintillation counting. As shown in Figure 5A, 340 counts were detected in the pyruvate fraction of a system containing 12 ng of the in vitro synthesized DAO. This value corresponded quite well to that obtained with the purified DAO from pig kidney.

When the spectrophotometric assay was used, the addition of D-alanine as substrate (first arrow on the right) resulted in a decrease in the absorbance at 340 nm, as shown in Figure 5B (the time course is from right to left). When sodium benzoate, a well-known potent competitive inhibitor of DAO, was added to the assay mixture, the rate of the decrease of absorbance at 340 nm was greatly decreased (Figure 5B). The apoenzyme synthesized in vitro in the absence of FAD exhibited almost the same catalytic activity when assayed in the presence of added FAD.

Kinetic Properties of the DAO Synthesized in Vitro. We investigated as to whether or not the characteristic kinetic properties of the authentic enzyme were shared by the in vitro translation product. Lineweaver–Burk plots of the catalytic reaction of the in vitro synthesized DAO based on the data obtained through the spectrophotometric measurements described above were linear (data not shown) with a Michaelis constant (K_m) for D-alanine of 2.56 mM and a maximum velocity of 3.85 μmol of D-alanine oxidized min^{-1} (mg of protein)$^{-1}$.

Discussion

Tissue-Specific Expression of DAO mRNA. Our previous study demonstrated the presence of three mRNA species for DAO in porcine kidney, due to the use of multiple polyadenylation signal sequences (ATTAAA and AACAAA) in the 3′-untranslated region. The efficiencies of the 3′ processing of RNA were quite different between the two signals, ATTAAA and AACAAA. This was also the case in liver but not in brain.

In brain, only one species of mRNA was detected. The presence of DAO activity in the brain had previously been reported (Gaunt & de Duve, 1976; Horiike et al., 1985). Our results suggest the active biosynthesis of this enzyme in brain and the presence of different processing machinery for DAO in porcine brain. Therefore, the regulatory mechanisms underlying DAO gene expression in brain may be quite different from those in other tissues. cDNA cloning for brain DAO and genomic sequencing are necessary for further analysis of the molecular mechanism regulating DAO gene expression.

No mRNA was detected in lung. This is in good agreement with the results of studies reported so far. It will be quite interesting to determine how the expression of the DAO gene is regulated during the differentiation of cells in each tissue.

In Vitro Synthesis of a Functional Enzyme by an SP6 Transcription System. In this paper, we describe the production of a functional DAO in vitro using a vector containing an SP6 promoter and DAO cDNA. It is now possible to clone and express a gene of interest in widely different host systems. We have described a novel system that is based on the high efficiency of in vitro transcription of the SP6 promoter (Melton et al., 1984; Krieg & Melton, 1984) and the high efficiency of translation in a rabbit reticulocyte lysate.

In the present study, the translation of RNA transcripts produced by the SP6 system directed the synthesis of a single protein species. No other small polypeptide derived from premature termination, nonspecific initiation at AUG codons, or degradation was detected.

Capping of RNA transcripts prior to translation significantly increased the efficiency of translation (18-fold). However, Spiess and Lodish (1986) did not observe improvement in protein synthesis with capping of the RNA in a wheat germ translation system. A 5′-cap structure was also reported to be quite essential for the stability of mRNA in oocyte nuclei (Green et al., 1983).

The in vitro synthesized DAO was indistinguishable from the authentic enzyme purified from porcine kidney by molecular weight, reactivity with the anti-DAO antibody, specific activity, kinetic parameters, and sensitivity to an inhibitor,

benzoate. Miyake et al. (1973) reported a Michaelis constant of 3.3 mM and a maximum velocity of 23.6 μmol of O_2 min^{-1} (mg of protein)$^{-1}$ for the authentic enzyme preparation, as judged by oxygraphy. Although the maximum velocity was relatively high, the assay was carried out in the absence of catalase and at 38 °C. Therefore, the kinetic parameters of the in vitro synthesized DAO seem to be comparable to those of the authentic enzyme. These results indicate that the cDNA we have cloned encodes all the information necessary for the expression of an apparently normal porcine kidney DAO, in the absence of any accessory molecules from the eukaryotic cells.

DAO has been shown to be synthesized on free ribosomes and to be transferred posttranslationally to the interior of peroxisomes without any proteolytic modification (Fukui et al., 1986, 1987). It will be of interest to determine whether FAD is incorporated into the DAO apoenzyme cotranslationally or posttranslationally in the process of its biosynthesis. Since our system can produce the apoprotein in vitro, the effect of FAD during the in vitro synthesis of DAO can be investigated. Preliminary results suggest that posttranslationally added FAD is effectively incorporated into the apoenzyme to yield a functional holoenzyme.

The availability of in vitro synthesized DAO should facilitate molecular studies on DAO biosynthesis, in studying the mode of FAD incorporation and translocation into peroxisomes. The present study also opens the way to molecular analysis of enzyme structure–function relationships and provides new insights into the mechanisms underlying enzyme catalysis.

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