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Comparison of Biochemical and Immunological Properties Between Rat and *Nicotiana glutinosa* Ornithine Decarboxylase

Yong-Sun Lee and Young-Dong Cho*
Department of Biochemistry, College of Science, Yonsei University, Seoul 120-749, Korea

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Ornithine decarboxylase (EC 4.1.1.17) is an essential enzyme for polyamine synthesis and growth in mammalian cells and plants. We compared the biochemical and immunological properties of rat and *Nicotiana glutinosa* ODC by cloning and expressing the recombinant proteins. The primary amino acid sequence between rat and *N. glutinosa* ODC had a 40% homology. The molecular weight of the overexpressed rat ODC was 53 kDa, and that of *N. glutinosa* was 46.5 kDa. Adding 1 mM of putrescine to the enzyme reaction mixture inhibited both rat and *N. glutinosa* ODC activity to 30%. Agmatine had an inhibitory effect only on *N. glutinosa* ODC. Cysteine and lysine modifying reagents reduced both ODC activities, verifying the key roles of cysteine and lysine residues in the catalytic mechanism of ODC. ELISA was performed to characterize the immunological difference between the rat and plant ODC. Both the rat and *N. glutinosa* ODC were recognized by the polyclonal antibody that was raised against purified *N. glutinosa* ODC, but the rat ODC was 50-fold less sensitive to the antibody binding. These results indicate that even though both ODCs have the same evolutionary origin, there seems to be a structural distinction between the species.

Keywords: *Nicotiana glutinosa*, Ornithine decarboxylase, Chemical modification, ELISA

Introduction

Polyamines are small polycations that are essential for cell growth and function. In cells, polyamine concentrations are highly regulated by the enzyme ornithine decarboxylase (ODC, EC 4.1.1.17), which catalyzes the conversion of ornithine to putrescine. Ornithine decarboxylase is a pyridoxal-5'-phosphate (PLP) dependent enzyme, and the decarboxylation reaction is the rate-limiting step in the biosynthesis of polyamines. The cloning and characterization of mammalian ODC genes from mice (Barabant *et al.*, 1988, Katz and Kahana, 1988), rats (van Steeg *et al.*, 1988; Wen *et al.*, 1989), and humans (Jeffery *et al.*, 1990) revealed that ODC is one of the most highly regulated enzymes (Seiler and Heby, 1988; Heby and Persson, 1990) with a short half-life. The regulation of ODC activity can be found at the transcriptional and translational levels, as well as at the level of mRNA and protein stability (Murasakami *et al.*, 1985; Katz and Kahana, 1987; Persson, 1988; Grens and Scheffler, 1990; Chen and Chen, 1992). Eukaryotic ODC consists of dimers of identical subunits that range from M_r 49,000-77,000 (McCann and Pegg, 1992; Tsirk and Coffino, 1992). The X-ray structure of ODCs from several eukaryotes, including mice and humans, is now available (Kern *et al.*, 1999). The catalytic roles of a number of active site residues have been reported (Osterman *et al.*, 1995; Osterman *et al.*, 1997; Osterman *et al.*, 1999). The N-terminal of the ODC enzyme folds in α/β-barrel domain and N-terminal domain contains a Lys-69 residue in the active site, which interacts with the PLP (Poulin *et al.*, 1992). The C-terminal domain of ODC folds into a β-barrel and contains residues, which have been identified to interact with the substrate. Cys-360 is the main residue that binds to the specific ODC inhibitor, α-difluoromethylornithine. In plants, ODC sequences from *Datura Stramonium* (GenBank™ X87847), *Lycopersicon esculentum* (GenBank™ AF029349) and *Nicotiana tabacum* (GenBank™ AB031066) are reported. Plants provide an interesting eukaryotic system for investigating the role of ODC and polyamines because an additional route to putrescine via arginine decarboxylase (ADC) is present (Tiburcio *et al.*, 1993). We were curious whether or not the plant ODC would be similar to other eukaryotic ODCs. We also wanted to know whether the presence of the additional ADC pathway meant that the plant ODC would have been under less selective pressure to conserve its primary structure.

In previous works we cloned and sequenced the *N. glutinosa* ODC and expressed the recombinant protein in *E.*

*To whom correspondence should be addressed.
Tel: 82-2-2123-2697; Fax: 82-2-313-1608
E-mail: cyd516@yonsei.ac.kr

coli (Lee and Cho, 2001). Based on these results, we compared the biochemical properties with rat ODC, which have not been studied in mammalian ODCs. In addition, a polyclonal antibody was raised against the purified *N. glutinosa* ODC. By using the *N. glutinosa* polyclonal antibody, we compared the structural difference between the rat and *N. glutinosa* ODCs. ELISA results provided compelling evidence that even though both ODCs had a similarity between the primary sequences that form the active site, there were structural differences between mammalian and plant species.

Materials and Methods

Materials Restriction enzymes and T4 DNA ligase were purchased from Promega. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) and ampicillin were purchased from Sigma. The *Nicotiana glutinosa* cDNA library was constructed by using a Stratagene uniZAP XR vector. Pfu DNA polymerase was also purchased from Stratagene. DNA sequencing was performed both with an automatic sequencer (ABI PRISIM 377 DNA Sequencer) and with the Sequenase 2.0 system from United States Biochemical. The pGEX-2T vector, glutathione, glutathione Sepharose 4B, thrombin, and L-[carboxy^{14}C]ornithine were obtained from Amersham Pharmacia Biotech. All of the oligonucleotides were acquired from TaKaRa Biomedicals. Anti-Rat IgG (H+L) alkaline phosphate conjugate were purchased from Promega. Gibco-BRL ELISA amplification System was used to amplify the amount of color generated by a given quantity of the alkaline phosphatase. All other reagents were obtained from commercial sources.

Cloning of *Nicotiana glutinosa* and rat ODC The *Nicotiana glutinosa* cDNA library and rat liver cDNA library was used to amplify ODC cDNA. The primers used for screening *N. glutinosa* were tODC(S) (5'-GGATCCATGGCCGGCCAAACAATAATCG TTTCCG-3') and tODC(A) (5'-CCCGGGTCAGCTTGGATAAGT ATAAGCGAGGTGAG-3'); for rat were rODC(S) (5'-GGATCCA TGGGCAGCTTTACTAAGGAAGAGTTTGA-3') and rODC(A) (5'-CCCGGGCTATACATTGATACTAGCAGAAGCACAGG-3'). Sense primers contained *BamHI* and antisense primers contained *SmaI* sites (underlined) to facilitate cloning (Sambrook *et al.*, 1989). The PCR reaction volume (50 µl) contained 500 nM primer each, 200 nM dNTPs, and 5 units of pfu DNA polymerase. The PCR reaction included an initial denaturation for 2 min at 94°C and then forty cycles of denaturation for 30 s at 94°C, annealing for 45 s at 53°C, and extension for 2 min at 72°C. The PCR product was digested with *BamHI* and *SmaI*. It was ligated to the *BamHI-SmaI* backbone fragment of pGEX-2T, which contained a T7 lac promoter and Glutathione-*S*-transferase preceding the N-terminus of the recombinant protein. The *E. coli* strain BL 21 (DE3) was transformed with the ligation product. Ampicillin-resistant transformants were selected, and plasmid DNA was purified from individual candidates. The identity of the cloned cDNA and the fidelity of the PCR were confirmed by DNA sequencing of the plasmid inserts by the dideoxy termination method (Sanger *et al.*, 1977) and an automatic sequencer (ABI PRISIM 377 DNA Sequencer).

Expression and purification of *N. glutinosa* and rat ODC *E. coli* BL 21 (DE3) was transformed with the pGEX2T-ODC plasmid. The transformants were tested for an ODC expression upon induction with IPTG. *E. coli* BL 21 (DE3) cells, carrying the expression plasmid that contained *N. glutinosa* and rat ODC, were grown overnight at 37°C in a LB medium containing 50 µg/ml ampicillin (Sambrook *et al.*, 1989). The cells were diluted 100-fold into the same medium and allowed to grow until A_{600} reached 0.5. To induce expression, 1 mM IPTG was added to the culture. The cells were harvested 4 h after induction by centrifugation (5,000 × *g*; 10 min) and sonicated in a Phosphate-buffered saline (PBS). The cell lysate was centrifuged at 13,000 × *g* for 20 min. The resulting supernatant was used for purification on the Glutathione-*S*-transferase Sepharose 4B resin in a batch procedure, according to the manufacturers recommendations. Recombinant *N. glutinosa* and rat ODC was recovered from the fusion protein by thrombin cleavage and then purified according to the manufacturers recommendations.

Protein analysis A SDS-polyacrylamide gel electrophoresis was performed by the Laemmli method (Laemmli, 1976) using a Tris-Glycine buffer [25 mM Tris-HCl, 200 mM, Glycine, 0.1% SDS (w/v), pH 8.3] and a 12.5% separating gel. Molecular masses of native enzymes were determined by a 5-20% polyacrylamide gradient gel electrophoresis. The protein concentration was determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

Enzyme activity assay The ODC activity was assayed at 37°C for 60 min by the liberation of $^{14}CO_2$ from L-[carboxy^{14}C]ornithine as a substrate (Kim and Cho, 1993). One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmole $^{14}CO_2$/h.

Chemical modification of ODC with group-specific reagents *N. glutinosa* and rat ODC were incubated with the sulfhydryl, carbonyl, lysyl and carboxyl group modification reagents. The 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), N-ethylmaleimide (NEM), *p*-chloromercuribenzoic acid (PCMB), salicylaldehyde, ethyldimethylamiopropylcarbodiimide (EDC), and phenylglyoxal were used in appropriate conditions (Means and Rober, 1971). Modification reactions were initiated by adding the reagent to the enzyme solution and then incubating it for 10 min at 25°C. After incubation, the aliquots of the reaction mixture that contained ODC were taken at the indicated time intervals and assayed. The control enzyme was subjected to the same treatment without the addition of the modification reagent. Before the sulfhydryl group modification reaction, dithiothreitol (DTT) was removed.

Antibody preparation The purified *N. glutinosa* ODC was used to immunize the rat. Then 200 µg of the enzyme, dissolved in PBS that contained 1 mM DTT, was emulsified with Freunds complete adjuvant and used for the first injection. A month later the rat was injected twice at intervals with 200 µg each of the enzyme in Freunds incomplete adjuvant. The rat was bled 10 days after the last injection. Two ml of antiserum was centrifuged and stored at –20°C.

ELISA analysis Flat-bottom 96 well plates were coated with

either 45 μg of purified *N. glutinosa* ODC, or rat ODC in PBS (pH 7.0) and incubated for 2 h at 37°C. After incubation, the plates were washed three times with a PBS buffer that contained 0.1% Tween-20 and blocked with 90 μl of 4% BSA-PBS for 1 h at 37°C. The plates were washed three times with PBS-T, then purified IgG for *N. glutinosa* ODC was added to each well. The antiserum was incubated for 1 h at 37°C. After washing the plates, goat anti-rat IgG that was conjugated with alkaline phosphatase was added to each well. Then 50 μl of the substrate, NADPH, was added to each well and incubated for 15 min at 25°C. Next, 50 μl of amplifier solutions (alcohol dehydrogenase and diaphorase) were added to each well. The change in absorbance was measured at 490 nm by using a microtiter plate spectrophotometer.

Results and Discussion

Expression and purification of *N. glutinosa* and rat ODC A lambda Uni-ZAP XR cDNA library of *N. glutinosa* was screened with the oligonucleotide pairs that represented conserved amino acid motifs of *N. tabacum* (GenBank™ AB031066) and other eukaryotic ODCs (Barabant *et al.*, 1988, Katz and Kahana, 1988). The rat liver cDNA library was also screened with the primers representing the 5' and 3' end of the rat ODC (van Steeg *et al.*, 1988; Wen *et al.*, 1989). The PCR product of *N. glutinosa* and rat ODC yielded a single band of 1299 bp (GenBank™ AF 323910) and 1386 bp, respectively. The deduced amino acid sequence, encoded by the open reading frame of *N. glutinosa* and rat ODC each, consisted of 432 and 461 amino acids. Comparison of the primary amino acid sequence of *N. glutinosa* and rat ODC showed a 40% homology between the two species (Fig. 1). *N. glutinosa* ODC lacked PEST-regions located at residues 423-449 in rat ODC. PEST regions are hydrophilic sequences (between positively charged residues) that are enriched in P, S/T or D/E that tend to reside near the carboxy terminal of enzymes (Coleman *et al.*, 1994). PEST regions act as a constitutive and conditional signal for the degradation of ODC by the proteasome system (Coleman *et al.*, 1994). Mammalian

Fig. 1. Comparison of the primary amino acid sequence of *N. glutinosa* and rat ODC. The *N. glutinosa* ODC sequence is from the nucleotide sequences submitted to the GenBank™/EMBL Data Bank with accession number AF32391. The rat ODC sequences were deduced from the papers describing the rat ODC (van Steeg *et al.*, 1988; Wen *et al.*, 1989). The sequence of N. glutinosa ODC (top) and rat ODC (bottom) are aligned. The secondary structures, predicted by PSIprep, are shown above and below the ODC sequences by H (α-helix) and E (β-sheet). The PEST regions of rat ODC are underlined.

Table 1. Effects of various polyamines on ODC activity. Enzymes were present at 1 mg/ml in PBS. Reactions were initiated by adding compounds to the enzyme and then incubating at 25°C for 10 min. After incubation, the aliquots (1 μg) were removed and the residual enzyme activity was assayed at 37°C for 1 h. The resulting activity was expressed as a percentage of that in the absence of any compound. ([a]Data from Lee and Cho, 2001)

Amine	Conc. (mM)	Relative ODC activity (%)	
		Rat	*N. glutinosa*[a]
Agmatine	0.1	107	39
	1.0	95	23
Cadaverine	0.1	108	85
	1.0	82	54
MTA	0.1	104	122
	1.0	30	84
Putrescine	0.1	122	64
	1.0	37	30
Spermidine	0.1	133	175
	1.0	99	211
Spermine	0.1	81	167
	1.0	86	309

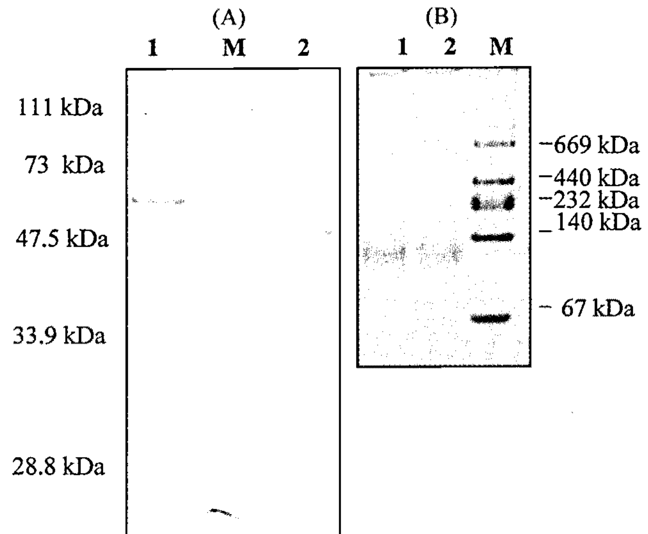

Fig. 2. PAGE-analysis of *N. glutinosa* ODC and rat ODC. (A) SDS-PAGE analysis of purified *N. glutinosa* ODC and rat ODC. *N. glutinosa* and rat ODC purification and thrombin proteolysis were performed, described in "Materials and Methods". The purified enzyme (5 mg) was analyzed by 12.5% SDS-PAGE. Lane 1, thrombin-cleaved rat ODC; lane 2, thrombin-cleaved *N. glutinosa* ODC (B) Polyacrylamide gradient gel electrophoresis analysis of purified *N. glutinosa* and rat ODC. *N. glutinosa* and rat ODC purification and thrombin proteolysis were performed, described in "Materials and Methods". The purified enzyme (5 mg) was analyzed by a 5-20% polyacrylamide gradient gel electrophoresis. Lane 1, thrombin-cleaved rat ODC; lane 2, thrombin-cleaved *N. glutinosa* ODC

ODC, lacking its PEST region that contains C-terminus, greatly reduces its access to the proteasome (Hayashi and Murakami, 1995; Hayashi *et al.*, 1996). Therefore, it seems possible that the *N. glutinosa* ODC activity is regulated *in vivo* by feedback inhibition of intracellular molecules, rather than by the proteasome 26S (Hayashi *et al.*, 1996). *N. glutinosa* ODC showed a single band of 46 kDa on the SDS-PAGE. Rat ODC showed a band of 53 kDa (Fig. 2A). The molecular mass of *T. brucei* (Phillips *et al.*, 1988), mouse (Barabant *et al.*, 1988, Katz and Kahana, 1988), and *Glycine max* (Kim and Cho, 1993) ODCs ranges from 45-55 kDa. This similar to *N. glutinosa* and rat ODC. Like other eukaryotic ODCs, *N. glutinosa* and rat ODC consists of dimers of identical subunits that form a homodimeric structure of 92 kDa and 106 kDa, respectively (Fig. 2B).

Effects of various amines on ODC We compared the effects of several amines on *N. glutinosa* and rat ODC activity. All of the amines, except for spermidine and spermine, inhibited *N. glutinosa* ODC activity. However, in rat ODC, MTA and putrescine inhibited the enzyme activity to 30% at the concentration of 1 mM (Table 1). Agmatine significantly inhibited the activities of SAMDC and ADC from the soybean (Yang and Cho, 1991). In the case of rat ODC, agmatine had no effect on the enzyme activity, but *N. glutinosa* ODC was inhibited to 23% by 1 mM agmatine. High levels of agmatine seemed to inhibit the ODC pathway, an alternative route for putrescine synthesis. Putrescine had an inhibitory effect on both rat and *N. glutinosa* ODC. Since putrescine is the decarboxylated product of L-ornithine, the ODC activity may be modulated *via* end-product inhibition.

Chemical modification of ODC A chemical modification were performed in order to compare the amino acid residues that are involved in the catalytic activity of *N. glutinosa* and rat ODC (Table 2). Sulfhydryl group-modifying reagents (such as NEM, DTNB, and PCMB) significantly inhibited both *N. glutinosa* and rat ODC activity. When the lysyl group modifying reagent, salicylaldehyde, was used, both ODC activities were inhibited, but it had a larger effect on *N. glutinosa* ODC. For a detailed study of salicylaldehyde, each enzyme was incubated with increasing concentrations of salicylaldehyde, ranging from 0.5 mM to 10 mM. This resulted in a rapid loss of *N. glutinosa* and rat ODC activity, while *N. glutinosa* was more sensitive to salicylaldehyde concentrations (Fig. 3). The carboxylgroup modification reagent, EDC, decreased both enzyme activities to a similar extent, but the arginine group modifying agent, phenylglyoxal, decreased the rat ODC activity by more than 2-fold at 1 mM concentrations. Figure 4 shows the DTNB mediated time dependent inactivation of rat and *N. glutinosa* ODC. DTNB was 20% more sensitive towards *N. glutinosa* ODC. By

Table 2. Effects of modification reagents on ODC. Enzyme was present at 1 mg/ml in PBS. Modification reactions were initiated by adding the reagent to the enzyme solution and incubating for 10 min at 25°C. After incubation, aliquots (1 μg) were removed and the residual enzyme activity was assayed at 37°C for 1 h. The resulting activity was expressed as a percentage of that in the absence of any compound. When sulfhydryl group modification reagents were tested, DTT was removed from the enzyme solution before incubation. ([a]Data from Lee and Cho, 2001).

Chemicals	Conc. (mM)	Relative ODC activity (%)	
		Rat	*N. glutinosa*[a]
DTNB	0.001	3.5	3.5
	0.01	1.9	0.9
NEM	0.001	1.7	1.3
	0.01	0.8	0.4
PCMB	0.001	2.9	2.4
	0.01	1.0	0.5
Salicylaldehyde	0.1	86	20.3
	1.0	18	1.7
EDC	0.1	82	54.8
	1.0	48	46.4
Phenylglyoxal	0.1	75	102
	1.0	29	63.7

adding excessive DTT, reactivation occurred in both of the enzymes. This result rules out the possibility that the loss of ODC activity is due to a conformational change by the modification reagents. The chemical modification results demonstrate that cysteine and lysine residues are highly involved in ODC activity, as reported from other eukaryotic sources (Kern *et al.*, 1999; Grishin *et al.*, 1999; Almrud *et al.*, 2000). The results indicated that *N. glutinosa* ODC was more susceptible to cysteine and lysine modification reagents.

ELISA analysis using *N. glutinosa* ODC antiserum Mammalian and plant ODC belongs to group IV carboxylases (Sandmeier *et al.*, 1994). They share a remarkable similarity between the amino acids that form the active site of ODC. Unlike mammalian ODC, there is no report on functionally important residues and the structural data on plant ODC. Therefore, to determine whether *N. glutinosa* and rat ODC differs in their protein surfaces and three-dimensional structure, the difference in the binding efficiency with the polyclonal antibody that was derived from purified *N. glutinosa* was measured. Figure 5 shows a decrease in the optical density at 490 nm by adding a serially diluted antiserum. There is a rapid decrease in the O.D. when rat ODC is used as the coated antigen, instead of the *N. glutinosa* ODC. The rat ODC showed about a 50 fold lower specificity to the plant ODC antibody. These results suggest that even

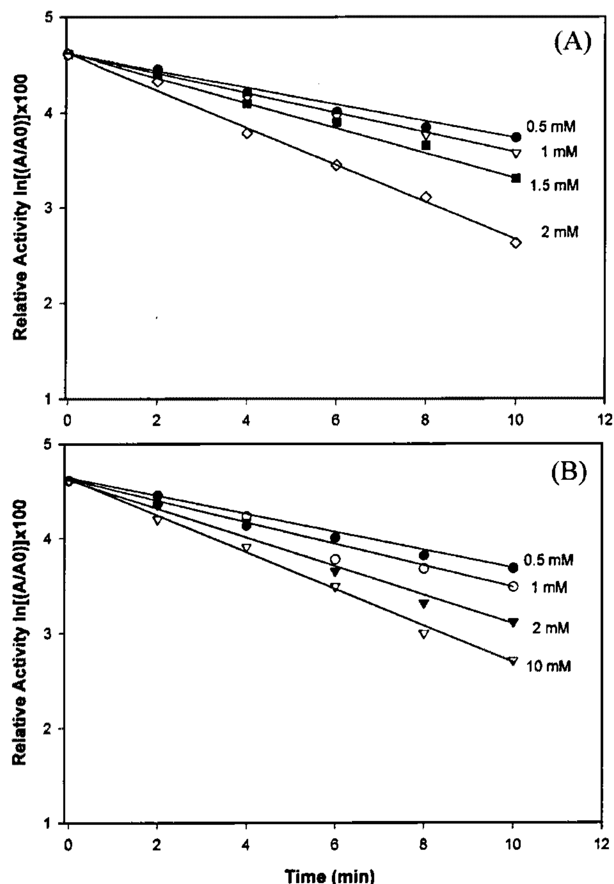

Fig. 3. Semilogarithmic plot for inactivation of *N. glutinosa* and rat ODC activity by salicylaldehyde. (A) Semilogarithmic plot for inactivation of *N. glutinosa* ODC activity by salicylaldehyde. The purified enzyme was incubated with various concentrations of salicylaldehyde in a phosphate-buffered saline (PBS) at 25°C for 10 min. After incubation, the aliquots (1 μg) were removed and assayed for residual activity in an assay buffer that contained 100 mM Tris-HCl, pH 8.0, 1 mM PLP, 1 mM DTT, and 50 μM EDTA. (A_0, original enzyme activity, A, enzyme activity at the time indicated) (B) Semilogarithmic plot for inactivation of rat ODC activity by salicylaldehyde. The purified enzyme was incubated with various concentrations of salicylaldehyde in a phosphate-buffered saline (PBS) at 25°C for 10 min. After incubation, the aliquots (1 μg) were removed and assayed for residual activity in an assay buffer that contained 100 mM potassium phosphate, pH 7.0, 1 mM PLP, 1 mM DTT, and 50 μM EDTA. (A_0, original enzyme activity, A, enzyme activity at time indicated)

though the mammalian and plant ODC have similar active site residues (cysteine and lysine), the overall three-dimensional structure of the protein differs to some extent. This difference was well distinguished by the sensitive immunological method of ELISA.

The secondary structure of the ODC monomers was presented (Fig. 1) and the tertiary structure of rat ODC was shown as ribbon diagrams (Fig. 6). Each monomer is comprised of two domains, referred to as the barrel domain

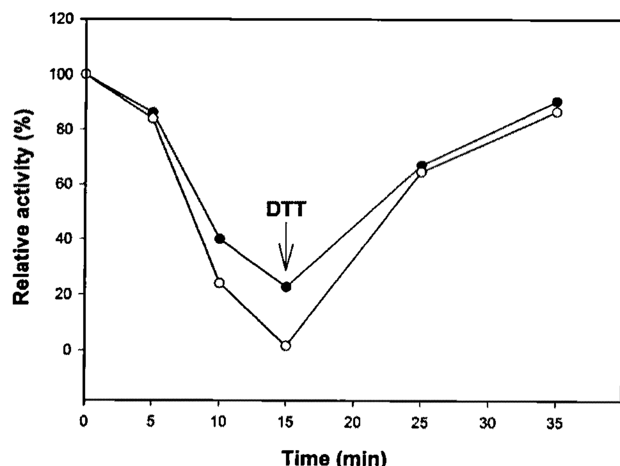

Fig. 4. Reactivation of the DTNB-inactivated *N. glutinosa* and rat ODC by excessive DTT. The purified *N. glutinosa* (m) and rat ODC (l) were incubated with 10 mM of DTNB at 25°C for the times indicated. After incubating for 15 min, 15 µl of 100 mM DTT were added to the reaction mixture to reactivate the enzyme. At various times, aliquots (1 µg) were removed and assayed for the ODC activity at 37°C for 1 h.

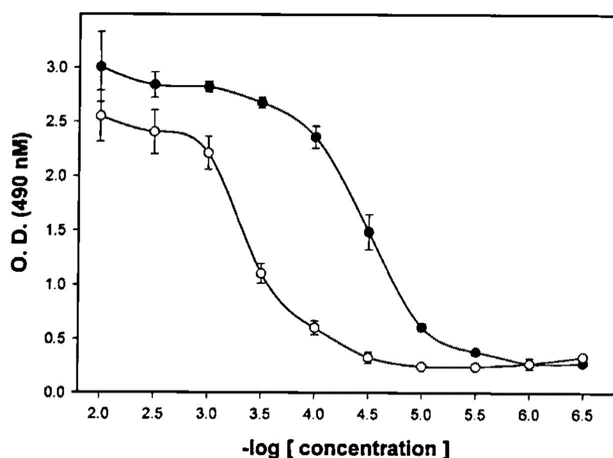

Fig. 5. Logarithmic titration curve for *N. glutinosa* and rat ODC with antiserum raised against purified recombinant *N. glutinosa* ODC 45 µg of purified *N. glutinosa* and rat ODC were coated onto Costar R. I. A. 96-well plate for 2 h at 37°C. After washing, serial dilutions of antiserums that contained an antibody raised against *N. glutinosa* ODC were added and incubated for 1 h. The antibody that was bound to *N. glutinosa* and rat ODC was detected with the goat anti-rat antibody that was conjugated with alkaline phosphatase. After washing, the bound enzyme activity was detected at 490 nm by using a microtiter plate spectrophotometer for *N. glutinosa* (l) and rat (m) ODC. Results are the mean ± standard deviation and are representative of the three experiments.

(shown in red) and the sheet domain (shown in light blue). The 3-dimensional homology model of the *N. glutinosa* ODC was compared with the rat ODC (unpublished observation). The overall structure was similar, but rat ODC had additional

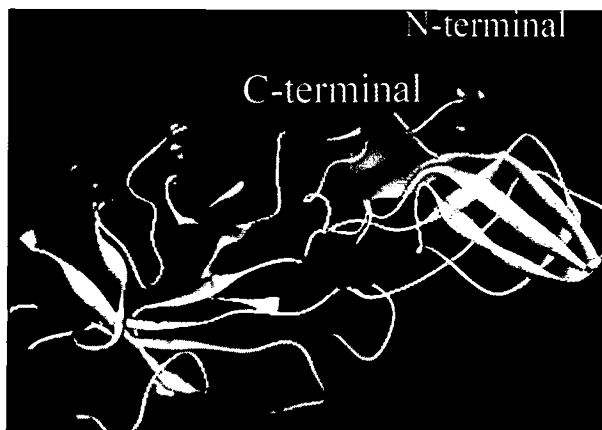

Fig. 6. The overall structure of rat ODC generated by molecular modeling. The overall three-dimensional structure of rat ODC are illustrated as ribbon diagrams. The helices and strands are indicated in red and light blue, respectively. These figures are drawn by a SWISS-MODEL, an automated comparative protein modeling server.

helices in the barrel and sheet domains. The active site of both ODCs was highly conserved. Each contains essential cysteine and lysine residues at the subunit interface. However, there were differences in the side chain orientation of several residues. The C-terminal tail of rat ODC has a basal degradation element that is required for proteolysis by the 26S proteasome, which is not found in *N. glutinosa*. The detailed conformational differences in the rat and *N. glutinosa* ODC should be determined by X-ray crystal structures of both ODC monomers and the ODC : antizyme heterodimers.

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