Identification of essential active-site residues in ornithine decarboxylase of *Nicotiana glutinosa* decarboxylating both L-ornithine and L-lysine

Yong-Sun LEE and Young-Dong CHO¹

Department of Biochemistry, College of Science, Yonsei University, Seoul 120–749, Korea

The cDNA encoding ornithine decarboxylase (ODC; EC 4.1.1.17), a key enzyme in putrescine and polyamine biosynthesis, has been cloned from *Nicotiana glutinosa* (GenBank® AF 323910), and was expressed in *Escherichia coli*. The amino acid sequence of *N. glutinosa* ODC showed 90 % identity with *Datura stramonium* ODC, and 44 % identity with human ODC. *N. glutinosa* ODC did not possess the PEST sequence [a sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues] found in mammalian ODCs, which are thought to be involved in rapid degradation of the protein. The purified ODC was a homodimeric protein, having a native M_r of 92 000. Kinetic studies of ODC showed that *N. glutinosa* ODC decarboxylated both L-ornithine and L-lysine with K_m values of 562 μ M and 1592 μ M at different optimal pH values of 8.0 and 6.8 respectively. ODC activity was completely and irreversibly inhibited by α -difluoromethylornithine (K_i 1.15 μ M), showing a competitive inhibition pattern. Site-directed mutagenesis was performed on ODC to introduce mutations at conserved lysine

(Lys⁹⁵) and cysteine (Cys⁹⁶, Cys³³⁸ and Cys³⁷⁷) residues, chosen by examination of the conserved sequence, which were proven by chemical modification to be involved in enzymic activity. Except for Cys⁹⁶, each mutation caused a substantial loss in enzyme activity. Most notably, Lys⁹⁵ increased the K_m for L-ornithine by 16-fold and for L-lysine by 3-fold, with 100-fold and 2.8-fold decreases in the k_{cat} for ODC and lysine decarboxylase (LDC) activity respectively. The Cys³⁷⁷ → Ala mutant possessed a k_{cat} that was lowered by 23-fold, and the K_m value was decreased by 1.4-fold for L-ornithine. The three-dimensional model of ODC protein constructed on the basis of the crystal structure of *Trypanosoma brucei*, mouse and human ODCs localized the four residues in the active-site cleft. This is the first work carried out on active-site residues of plant ODC, where ODC and LDC activities occur in the same catalytic site.

Key words: polyamine, putrescine, pyridoxal 5-phosphate (PLP), site-directed mutagenesis.

INTRODUCTION

Ornithine decarboxylase (ODC; EC 4.1.1.17) is an important rate-limiting enzyme in polyamine biosynthesis [1]. Putrescine, the product of ODC catalysis, and the subsequent metabolic pathway products, spermidine and spermine, are ubiquitous to all cells and are required for cell growth and differentiation. The activity of ODC is very rapidly and greatly changed in response to stimuli affecting cell growth and polyamine content, and the underlying biochemical mechanisms have been studied extensively [2,3]. ODC was identified as a drug target against certain cancers and parasitic diseases [4,5], and X-ray structures of parasite *Trypanosoma brucei* and their host, mouse and human ODCs are now available for the development of novel therapeutic agents [6–8]. These ODCs have a conserved Lys⁶⁹ residue, which interacts with pyridoxal 5-phosphate (PLP) [9], and the positive charge on the pyridine nitrogen of PLP is stabilized by the negative charge of the Glu²⁷⁴ residue [10]. In *T. brucei* ODC, Arg²⁷⁷ is required for high-affinity PLP binding via interaction with the 5'-phosphate of the cofactor [11]. The C-terminal domain contains residues that have been identified to interact with the substrate, and Cys³⁶⁰ is the main residue that binds to the specific ODC inhibitor, α -difluoromethylornithine (α -DFMO).

Despite extensive work on ODCs, the kinetic and catalytic mechanisms of plant ODC remains unclear. Plants provide an interesting eukaryotic system for investigating the role of ODC and polyamine because of an additional pathway leading to putrescine via arginine decarboxylase (ADC), as found also in

bacteria [12,13]. Bacterial ODC genes are unrelated to the animal and fungal ODCs that synthesize putrescine without an additional ADC pathway. Therefore we were interested in investigating whether *Nicotiana glutinosa* ODC is similar to other eukaryotic ODCs, or whether the presence of an additional ADC pathway led to less stringency in terms of conservation of the gene/protein structure of ODC. An additional point of interest relating to *N. glutinosa* ODC was its decarboxylation activity towards both L-ornithine and L-lysine. There have been previous reports on ODCs from two parasites, *T. brucei* and *Leishmania donovani*, showing that decarboxylation activity occurs towards L-ornithine, L-lysine and L-arginine [10]. The ODC of an anaerobic protozoan, *Trichomonas vaginalis*, also decarboxylated L-lysine at slow rates [14]. In mammals, similar results have been reported for mouse kidney, rat liver and prostate ODCs, showing that these possess lysine decarboxylase (LDC) activity in addition to that of ODC [15,16]. On the other hand, *Selenomonas ruminantium* LDC was reported to have decarboxylase activities towards both L-lysine and L-ornithine, with similar K_m values [17]. Although structural and kinetic studies have revealed many details about the precise roles of active-site residues in mammalian and *T. brucei* ODCs, there have been no reported studies on plant ODCs, except for one report on the cloning of several plant genes.

In the present paper we describe cloning and expression studies on recombinant wild-type and site-directed mutants of *N. glutinosa* ODC for the first time in plants. Determination of the kinetic parameters for wild-type recombinant ODC shows that these are essentially identical with those of the native enzyme,

Abbreviations used: ADC, arginine decarboxylase; C377A, mutant bearing the site-directed mutation of Cys³⁷⁷ → Ala, etc.; DFMO, α -difluoromethylornithine; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GST, glutathione S-transferase; IPTG, isopropyl β -D-thiogalactoside; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; PLP, pyridoxal 5-phosphate.

¹ To whom correspondence should be addressed (e-mail cyd516@yonsei.ac.kr).

and a single polypeptide is responsible for both LDC and ODC activity. In order to analyse the roles of the active-site residues in catalysis, we have examined the effects of the conserved residues by mutating the conserved lysine residue Lys⁹⁵ and cysteine residues Cys⁹⁶, Cys³³⁸ and Cys³⁷⁷ into alanine. Chemical modification and site-directed mutagenesis of the conserved amino acids in *N. glutinosa* ODC provides definitive evidence for both ODC and LDC activities being mediated via one active site in a single polypeptide. In addition, a comparative model, which is the first molecular model of *N. glutinosa* ODC, is presented to predict the catalytic fold. The catalytic fold of *N. glutinosa* ODC was predicted to be similar to that of *T. brucei*, human and mouse ODCs [6–8]. In this model, Lys⁹⁵, Cys⁹⁶ and Cys³⁷⁷ are positioned as a catalytic triad in the active site, and Cys³³⁸ is predicted to be a further important residue in substrate binding, a residue not studied previously. Determination of the active-site fold is the first step towards defining the structure of plant ODC, providing a basis for the development of specific regulators that can be used to understand the role of this enzyme in plants.

MATERIALS AND METHODS

Materials

Restriction enzymes and T4 DNA ligase were purchased from Promega. Isopropyl β -D-thiogalactoside (IPTG) and ampicillin were purchased from Sigma. The *N. glutinosa* cDNA library was constructed by using a Stratagene (La Jolla, CA, U.S.A.) uniZAP XR vector. *Pfu* DNA polymerase was also purchased from Stratagene. DNA sequencing was performed with both an automatic sequencer (ABI PRISM 377 DNA Sequencer) and the Sequenase 2.0 system from United States Biochemical Corp. (Cleveland, OH, U.S.A.). pGEX-2T vector, glutathione, glutathione–Sephadex 4B, thrombin, L-[U-¹⁴C]lysine and L-[carboxy-¹⁴C]ornithine were obtained from Amersham Pharmacia Biotech. All oligonucleotides were acquired from TaKaRa Biomedicals (Kyoto, Japan). All other reagents were obtained from commercial sources.

Cloning of ODC cDNA and site-directed mutagenesis

ODC cDNA was amplified by PCR using the *N. glutinosa* cDNA library. The oligonucleotides contained *Bam*HI and *Sma*I sites to facilitate cloning [18]. The PCR reaction vol. (50 μ l) contained 500 nM of each primer, 200 μ M dNTPs and 5 units of *pfu* DNA polymerase. The PCR reaction included an initial denaturation step for 2 min at 94 °C, and then 40 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 *Bam*HI and *Sma*I, and ligated into the *Bam*HI–*Sma*I backbone fragment of pGEX-2T, which contains T7lac promoter and glutathione S-transferase (GST) preceding the N-terminus of the recombinant protein. *Escherichia coli* strain BL21(DE3) was transformed with the ligation product. Ampicillin-resistant transformants were selected, and plasmid DNA was purified from individual colonies. The identity of the cloned cDNA and the fidelity of PCR were confirmed by DNA sequencing of the plasmid inserts by the dideoxy termination method [19] and automatic sequencing (ABI PRISM 377 DNA Sequencer). Ten oligonucleotides were designed to replace amino acid residues potentially involved in ODC activity, which were chosen by examination of the conserved sequences of ODC (see Figure 1) and chemical-modification experiments. Mutations resulting in the amino acid substitutions Lys⁹⁵ $\rightarrow$ Ala (K95A) and C96A were introduced by sequential PCR [20], and C338A and C377A mutations were created by using the Quick Exchange™ site-directed mutagenesis

kit from Stratagene, according to manufacturer's instructions. Terminal sense and antisense primers were designed to hybridize with the N-terminal and C-terminal regions respectively, with the introduction of restriction sites (*Bam*HI in the sense primer and *Sma*I in the antisense primer) to facilitate ligation and cloning. For sequential PCR, internal primer sets were designed to hybridize with the regions flanking the mutation site. The internal antisense primer has a mutated codon sequence, and more than half of the sequence of this primer is complementary to the internal sense primer (Table 1). In the first reaction, either terminal sense and internal antisense primers or internal sense and terminal antisense primers were used as a primer set, and a recombinant pGEX-2T plasmid containing the *N. glutinosa* ODC cDNA sequence was used as a template. PCR reaction conditions were as follows (a total of 30 cycles): initial denaturation for 10 min at 94 °C, then 45 s at 94 °C, 45 s at 53 °C, and 2 min at 72 °C. In the second round of reactions, terminal sense primer and terminal antisense primer were used as a primer set, and two PCR products from the first-round reactions were used as templates to produce a single annealed PCR product. The conditions for PCR were as follows: initial denaturation for 2 min at 94 °C, proceeded by 30 cycles of denaturation for 1 min at 94 °C, annealing for 45 s at 53 °C and a final extension reaction for 2 min at 72 °C. The final PCR product was digested with *Bam*HI and *Sma*I, before ligation into the pGEX-2T vector linearized with *Bam*HI and *Sma*I and dephosphorylation with calf intestinal alkaline phosphatase. This ligation mixture was used to transform *E. coli* BL21(DE3). Verification of all mutants was achieved by performing DNA sequencing of plasmid inserts by the dideoxy termination method [19].

Expression and purification of wild-type and mutant ODC

E. coli BL21(DE3) was transformed with the pGEX2T-ODC plasmid, and transformants were tested for ODC expression upon induction with IPTG. *E. coli* BL21(DE3) cells carrying the expression plasmid containing wild-type or mutant ODC were grown overnight at 37 °C in Luria–Bertani medium (containing 50 μ g/ml ampicillin) [18]. The cells were diluted 100-fold into the same medium, and allowed to grow until a D_{600} of 0.5 was attained. To induce expression, 1 mM IPTG was added to the culture. The cells were harvested 4 h after induction by centrifugation (5000 *g* for 10 min) and then sonicated in PBS, before centrifugation of the cell lysate at 13000 *g* for 20 min. The resulting supernatant was used for purification on glutathione–Sephadex 4B resin in a batch procedure, according to the manufacturer's recommendations. Recombinant mutated or wild-type ODC was recovered from the fusion protein by thrombin cleavage, and then purified according to manufacturer's recommendations. The purity of the recombinant protein was assessed by SDS/PAGE and polyacrylamide gradient gel electrophoresis [21].

Enzyme activity assay

ODC activity was assayed in 40 mM Tris/HCl buffer, pH 8.0, containing 1 mM dithiothreitol and 1 mM PLP at 37 °C for 60 min by the liberation of ¹⁴CO₂ from 0.1 μ Ci of L-[carboxy-¹⁴C]ornithine mixed with unlabelled L-ornithine to a final concentration of 400 μ M [22]. LDC activity was assayed similarly to the ODC activity, except that 0.5 μ Ci of L-[U-¹⁴C]lysine was used instead of L-[carboxy-¹⁴C]ornithine in 40 mM of potassium phosphate buffer, pH 6.8. ODC and LDC activity was a linear function of both incubation time and concentration under these conditions. One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 nmol of ¹⁴CO₂/h.

	B1-bb	B2-bbbb	H1-hhhhhhhhhhhhhhh	B3-b	H2-hhhhhhh				
<i>N. glutinosa</i>	MAGQTIIVSGLNPAAILQSTIGGGASPTAAAAENGTRKVIPLSRDALQDFMLSIITQKLQDEKQPFYVLDLGEVVSLLMDQ					80			
<i>D. stramonium</i>	MAGQTIIVSGLNPAAILQSTIGG-ATPAPAAENDHTRKVVPLSRDALQDFMVSIIITQKLQDEKQPFYVLDLGEVVSLLMDQ					79			
<i>T. brucei</i>	-----RDALCKKISMNTCDEGDPFFVADLGDIIVRKHET					74			
human	-----DDKDAFYVADLGDIILKKHLR					54			
mouse	-----DDKDAFYVADLGDIILKKHLR					54			
	↓ ↓								
	hhhh	B4-bbbb	H3-hhhhhhh	B5-	H4-hhhhhhh	B6-bb	H5-hhhhhhh	B7-b	
<i>N. glutinosa</i>	WKSSLPNIRPFYAVKCNPEPSFLSILSAMGSNEDCASRAETFEYVLALGISPDRIVFANPCKPESDIIFAAKVGVNLTTFD								160
<i>D. stramonium</i>	WNAGLPNIRPFYAVKCNPEPSFLSILSAMGSNEDCASRAETFEYVLALGISPDRIVFANPCKPESDIIFAAKVGVNLTTFD								159
<i>T. brucei</i>	WKKCLPRVTPEFYAVKCNDDWRVLGTLAALGTCFDCASNTETQVRVGIGVPEKIIYANPCKQISHIRYARDSGVDMVTFD								154
human	WLKALPRVTPEFYAVKCNDSKAIKTLAATGTGFCASKTEIQLVQSLGVPPERIIYANPCKQVSIKYAANNQVMVTFD								134
mouse	WLKALPRVTPEFYAVKCNDSRAIVSTLAAIGTCFDCASKTEIQLVQSLGVPAERVIIYANPCKQVSIKYAASNGVQMMTFD								134
	H6-hhhhhhh	B8-bbbb	H7-hhhhhhhhh	B9-bbbbb	H8-hhhhh				
<i>N. glutinosa</i>	SEDEVYKIRKHHPKSELLPRIKPMFDGNARCPMGPKYCALPEEVEPLLRAAQAARLTVSGVSTFHGSGDADSNAYLGATA								240
<i>D. stramonium</i>	SEDEVYKIRKHHPKCELLLRKPMDDGNARCPMGPKYCALPEEVEPLLRTAQAARLTVSGVSTFHGSGDADSKAYLGATA								239
<i>T. brucei</i>	CVDELEKVAKTHPKAKMVLRISTD-DSLARCRLSVKFGAKVEDCRFILEQAKKLNIIDVTGVSTFHVSGSGTDASTFAQAIS								233
human	SEVELMKVARAHPKAKLVLRITATD-DSKAVCRLSVKFGATLRLSRLLERAKELNIDVVGVSFHVSGSGCTDPETVQVAIS								213
mouse	SEIELMKVARAHPKAKLVLRITATD-DSKAVCRLSVKFGATLRLSRLLERAKELNIDVIGVSFHVSGSGCTDPDPTVQVAIS								213
	hhhhhhhhhh	B10-	H9-hhhhhhhhh	B11-b	H10-	B12-bbbbbbb			
<i>N. glutinosa</i>	AAKEVFETAAKLGMSKMTVLDDVGGGFTSGHQFTTAAVAVKSALEKQHFDEPELTIIAEPGRFFAETATTLATTVIGKRV								320
<i>D. stramonium</i>	AAKGVEETAAKFGMSKMTVLDDVGGGFTSGHQFTTASAAVRSALQHFHDEQELTIIAEPGRFFAETATTLATTVIGKRV								319
<i>T. brucei</i>	DSRGVEFDMGTGLFN-MHLLDIGGGFPGTRKFEEIAGVINNALEKHFPPDLKLTIVAEPPGRYYVASAFTLAVNVIKKT								316
human	DARCVFDMGAEVGF-SMYLLDIGGGFPGSEKFEETIGVINPALDKYFSPDSGVRIIAEPGRYYVASAFTLAVNIIAKKTE								299
mouse	DARCVFDMATEVGF-SMYLLDIGGGFPGSEKFEETISVINPALDKYFSPDSGVRIIAEPGRYYVASAFTLAVNIIAKKTE								299
	↓								
	B13-bbb		B14-bbbb	B15-b		B16-			
<i>N. glutinosa</i>	GELREYVINDGLYGSNNCVLYDHA TVNATPLAVLSNRTNVTGGSKTFPTTVFGPTCDALDVLDRDYQLPELQVNDWLVF								400
<i>D. stramonium</i>	GELREYVINDGLYGSNNCVLYDHA TVNATPLACMSNRSLNCGSKTFPTTVFGPTCDALDVLDRDYQLPELQVNDWLIF								399
<i>T. brucei</i>	GAHAEEYVNDGVYGSFNCILYDHA VVRPLP-----QREPI--PNEKLYPSSVWGPTCDGLDQIVERYYPENQVGEWLLF								403
human	DESSEYVNDGVYGSFNCILYDHA HVK--PL-LQKRPKPD---EKYSSSIWGPTCDGLDRIVERCDLPEMHVGDWMLF								383
mouse	DESNEYVNDGVYGSFNCILYDHA HVKA--LQKRPKPD---EKYSSSIWGPTCDGLDRIVERCNLPEMHVGDWMLF								383
	b	B-17	B18-bbb						
<i>N. glutinosa</i>	PNMGAYTKAAGSNFNGENTSTIVTHLAYTYS								432
<i>D. stramonium</i>	PNMGAYTKAAGSNFNGENTSAIVTHLAYAYS								431
<i>T. brucei</i>	EDMGAYTVVGTSSNFGFQSPTI-----								425
human	ENMGAYTVAAASTFNCFORPTI-----								405
mouse	ENMGAYTVAAASTFNCFORPNI-----								405

Figure 1 Comparison of the primary amino acid sequence of *N. glutinosa*, *D. stramonium*, *Trypanosoma brucei*, human and mouse ODCs

Sequences were deduced from the references describing *N. glutinosa* (GenBank® AF323910), *D. stramonium* (GenBank® X87847), *T. brucei* (GenBank® J02771), human (GenBank® J02771) and mouse. Regions of primary structure similarity are enclosed in grey-shaded boxes, and the arrows indicate the amino acid residues in the *N. glutinosa* sequence that were mutated, as described in the Materials and methods section. The secondary-structural elements of *N. glutinosa* ODC are shown above the sequence (B1–B18, β -sheets 1–18 respectively; H1–H10, α -helices 1–10 respectively).

Table 1 Mutagenic oligonucleotides for site-directed mutagenesis of ODC

Nucleotides that were exchanged in order to obtain the desired mutation are shown underlined in bold letters. Numbers in superscript indicate the position of nucleotides in the coding region of the gene. Restriction sites are underlined (sense; *Bam*HI; antisense; *Sma*I). Mutations at a specific residue number are indicated by one letter amino-acid abbreviations. The first letter is the wild-type (WT) residue, and the last letter is the amino acid to which it is changed by the mutated codon. WT, wild type; S, sense; AS, antisense.

Nucleotide sequence	Oligonucleotide
5'-GGATCCATGGCCGGCCAAACAATAATCGTTTCG	Sense
5'-CCCAGGTCAGCTTGATAAGTATAAGCAGGTGAG	Antisense
5'- ²⁶³ TTACGCTGTTAAATGTAACCGTGAACCGTC	K95WT
5'-TTACGCTGTTGATGTAACCGTGAACCGTC	K95A (S)
5'-CGACGGTTCAGGGTTACATGCAACAGCGTA	K95A (AS)
5'- ²⁶⁴ TACGCTGTTAAATGTAACCGTGAACCGTC	C96WT
5'-TACGCTGTTAAAGCTAACCGTGAACCGTC	C96A (S)
5'-CGACGGTTCAGGGTTAGCTTAAACAGCGT	C96A (AS)
5'- ⁹⁹⁹ CGGTTTCGATGAACCTGCGTACTTTACGAC	C338WT
5'-CGGTTTCGATGAACCGCTGACTTTACGAC	C338A (S)
5'-GTCGTAAGTACGCGGTTTCATCATGAACCG	C338A (AS)
5'- ¹¹²⁰ GGGCCACCTGTGATGCTCTTGATACTG	C377WT
5'-GGGCCACCGCTGATGCTCTTGATACTG	C377A (S)
5'-CAGTATCAAGAGCATCAGCGGTGGGCC	C377A (AS)

Chemical modification of ODC with group-specific reagents

Wild-type ODC was incubated with the thiol-group modification reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [23]. After incubating at 25 °C for the various times indicated (see Figure 4), modified enzymes were applied to Centricon to remove the unreacted DTNB, as described in the instructions for the Millipore kit (Bedford, MA, U.S.A.). Of PBS-washed enzyme, 1 μ g was added to the reaction mixture and incubated at 37 °C for 60 min. The control enzyme was subjected to the same conditions without adding the modification reagent.

Kinetic characterization of wild-type and mutant ODCs

Substrate saturation studies of the wild-type and mutant ODC activity were performed to determine K_m and V_{max} values for L-ornithine and L-lysine. The initial rates were determined by plotting the amount of product against time. The K_m , V_{max} and k_{cat} values were calculated from plots of $1/V_i$ against $1/[S]$ (where V_i represents initial velocity and $[S]$ is the substrate concentration) [24]. K_i values of various concentrations of L-ornithine (from 200 μ M to 1.5 mM) were examined in the presence of 0.5 μ M or 1 μ M α -DFMO, an irreversible inhibitor of ODC. After

incubation at 25 °C for 10 min, the modified enzymes were applied to the Centricon to remove unreacted α -DFMO. Of the PBS-washed enzyme, 1 μ g was added to the reaction mixture and incubated at 37 °C for 60 min.

Optimal pH and thermal stability

The optimum pH of the ODC enzyme was determined by incubating the reaction mixture in 40 mM potassium phosphate buffer (pH 6.4–7.6) or 40 mM Tris/HCl buffer (pH 7.2–8.6) at 37 °C for 60 min. The optimal temperature of the wild-type ODC was measured as described previously [22,25], and thermal stability of the ODC was measured by incubating PBS-diluted enzymes (1 mg/ml) at 40, 50 and 60 °C. At various times, aliquots were removed and assayed for the residual ODC activity. The control sample of enzyme was incubated on ice as a reference.

CD measurements

Spectral acquisition was performed on a Jasco 720 Spectropolarimeter (Japan Spectroscopic Ltd., Tokyo, Japan). Three scans were made at 20 nm/min between 260 and 200 nm in a 0.1 mm path length and a volume of 300 μ l at 25 °C. After subtraction of the buffer spectra, the data were converted into molecular ellipticity units. Protein secondary structure was calculated from the Jasco secondary estimation program [26].

Prediction of secondary and tertiary structures of *N. glutinosa* ODC

In order to obtain a prediction of the secondary structure, the sequence of *N. glutinosa* ODC was submitted to PSIPred (a collection of protein structural prediction methods at the University of Warwick) [27]. For three-dimensional structure prediction, the *N. glutinosa* ODC sequence was sent to Swiss-Model, an automated comparative protein modelling server (<http://www.expasy.ch/swissmod/SWISS-MODEL.html>), which runs on the software ProMod II and Gromos96 [28]. The tertiary structure data of mouse ODC [6] and *T. brucei* ODC [7] were used as the template for the prediction. The three-dimensional structure was visualized with the software POV-Ray for Windows and Swiss-Protein Data Bank (PDB) Viewer 3.51.

RESULTS AND DISCUSSION

Cloning and expression of wild-type and mutant ODCs

A λ Uni-ZAP XR cDNA library of *N. glutinosa* was screened with the oligonucleotide pairs representing conserved amino acid motifs of *N. tabacum* and other eukaryotic ODCs. The PCR product yielded a single band of 1299 bp and the deduced amino acid sequence encoded by the open reading frame of *N. glutinosa* ODC consists of 432 amino acids (GenBank® accession no. AF323910). *N. glutinosa* ODC showed 98 % and 90 % identity with *N. tabacum* and *Datura stramonium* respectively, and 43 % (*T. brucei*), 45 % (yeast), 43 % (mouse) and 44 % (human) identity with other eukaryotes over the common core regions (Figure 1). Oligonucleotides were designed to replace amino acid residues potentially involved in ODC activity, chosen on the basis of chemical-modification experiments and by examining sequences conserved in other eukaryotes. Three conserved putative active-site cysteines, Cys⁹⁶, Cys³³⁸ and Cys³⁷⁷, and Lys⁹⁵ were changed to alanine. By using the pGEX-2T GST-fusion vector, we obtained high levels of recombinant protein in soluble forms. Treatment of the GST–ODC fusion protein with thrombin, and subsequent GST–Sepharose 4B affinity column chromatography, yielded a single band of 46 kDa on an SDS/12.5 % (w/v)

Figure 2 SDS/PAGE and polyacrylamide gradient gel electrophoretic analysis of purified *N. glutinosa* ODC

(A) Wild-type and mutant ODC purification and thrombin proteolysis were performed as described in the Materials and methods section. The purified enzyme (5 μ g) was analysed by SDS/PAGE (12.5 % gels). (B) Wild-type and mutant ODC purification and thrombin proteolysis were performed as described in the Materials and methods section. The purified enzyme (5 μ g) was analysed by 5–20 % polyacrylamide gradient gel electrophoresis. For both gels, lanes were loaded as follows: lane 1, molecular mass markers; lane 2, thrombin-cleaved wild-type ODC; lane 3, thrombin-cleaved K95A ODC; lane 4, thrombin-cleaved C96A ODC; lane 5, thrombin-cleaved C338A ODC; lane 6, thrombin-cleaved C377A ODC.

polyacrylamide gel (Figure 2A). The molecular mass determined from the gel was in good agreement with the value of 46.5 kDa calculated for the 432-amino-acid protein. *N. glutinosa* ODC is slightly larger than the *T. brucei* ODC, which has a molecular mass of 45 kDa [29], but smaller than the mouse (53 kDa) [30] or *Glycine max* ODC (55 kDa) [22]. The composition of the charged amino acid residues was nearly identical with that of mouse ODC, which was reflected by similar calculated isoelectric points of pI 5.48 for *N. glutinosa* ODCs and pI 5.07 for mouse ODC. The native molecular mass of the protein was 92 kDa, estimated from PAGE (Figure 2B), indicating that *N. glutinosa* ODC has a homodimeric structure.

Eukaryotic ODCs consist of dimers of identical subunits of M_r 49 000–77 000 [31,32], and are dependent on PLP for activity. The predicted amino acid sequences of *N. glutinosa* ODC and other eukaryotic ODCs belong to group IV PLP-dependent decarboxylases [33]; there is extensive similarity among these enzymes. The similarity among *N. glutinosa*, *D. stramonium* and mouse ODC amino acid sequences permits Lys⁹⁵ to be assigned as part of the PLP-binding site, and Cys³⁷⁷ to be assigned as the DFMO-binding site on the *N. glutinosa* enzyme. Moreover, the *N. glutinosa* ODC is suggested to be active as a homodimer on the basis of the presence of the conserved residue Gly⁴⁰⁴, which plays an essential role in dimer formation in mouse ODC [34]. This was verified by our PAGE data, presented in Figure 2(B). Unlike

Figure 3 pH profiles for ODC and LDC activities of ODC

The purified enzyme was assayed for ODC or LDC activity in various ranges of pH buffers, as described in the Materials and methods section. The LDC activity was assayed in 40 mM potassium phosphate buffer (○), and the ODC activity in 40 mM Tris/HCl buffer is also shown (■).

mammalian ODC, there is no C-terminal PEST sequence [a sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues] in *N. glutinosa* ODC, which plays an important role in rapid turnover *in vivo* [35]. It is therefore possible to predict that *N. glutinosa* ODC is more stable than mammalian proteins, as reported for *D. stramonium* [36] and *T. brucei* [37].

Substrate specificity for L-ornithine and L-lysine

The purified recombinant wild-type ODC showed its highest catalytic activity towards L-ornithine at pH 8.0. Interestingly, *N. glutinosa* ODC showed LDC activity in the pH range from 6.5 to 7.0, with its pH optimum at 6.8, lower than that of the ODC activity (Figure 3). The optimal pH of ODC was determined in the range of pH from pH 6.5 to 8.5. At the optimal pH of ODC

activity, very low LDC activity was measured. However, a shift from alkaline pH to acidic pH caused a rapid decrease in the activity of ODC, while LDC activity increased rapidly (Figure 3). Activities of ODC towards L-ornithine and L-lysine were therefore largely dependent on pH. Hence *N. glutinosa* ODC, like those of other eukaryotes, such as *T. vaginalis* [14], rat [15] and the mouse (derived from kidney) [37], can decarboxylate lysine at a slow rate. It appears that the ODCs from eukaryotes, including plants, have substrate specificity towards both L-ornithine and L-lysine, whereas prokaryotes have separate enzymes to decarboxylate basic amino acids with strict specificities [38]. For the ODCs from rat liver, prostate and kidney, broad substrate specificity was observed at the same pH values [15], showing ODC activities in the range of nmol of $^{14}\text{CO}_2$ /mg of protein per h, and LDC activities in the range of pmol of $^{14}\text{CO}_2$ /mg of protein per h. This low activity of LDC in rat ODC might have been due to the high pH (pH 7.5) used in the assay buffer, which could be sub-optimal for the LDC activity.

The rate of enzyme action on a mixture of two substrates compared with that on the two substrates presented singly has been used to determine whether two reactions are due to the same enzyme [39]. An experiment of this kind was performed with the mixed substrate of L-ornithine and L-lysine. Mixed-substrate experiments have most commonly been performed with the two substrates in equimolar concentrations. In the present study, 400 μM L-ornithine and 1.2 mM L-lysine was used, showing their relative concentrations (that is, the ratio of their concentrations to the respective Michaelis constants) of 0.7 and 0.753 respectively. If one enzyme is involved in two reactions, the mixed substrate velocity can be expressed by using the equation $v_t = (V_a \alpha V_b \beta) / (1 + \alpha + \beta)$ [39,40]. V_a and V_b represent the maximum velocities with an excess of two substrates, L-ornithine and L-lysine, and v_t is the mixed-substrate velocity when the relative concentrations are α and β . In this experiment, the calculated v_t value over the sum of the velocities with the substrate attacked separately was 0.28, and the experimental data in Table 3 show a similar ratio of 0.26 for the observed mixed rate over the sum of the observed individual rates, suggesting a single enzyme. Comparison of the specificity constant (k_{cat}/K_m) revealed that *N. glutinosa* ODC exhibited a preference for L-ornithine over

Table 2 Kinetic analysis of *N. glutinosa* ODC mutants by using L-ornithine or L-lysine as the substrate

(A) Wild-type (WT) and mutant ODCs were purified as described in the Materials and methods section, and K_m , V_{max} and k_{cat} values were determined from double-reciprocal plots of [L-ornithine] against initial velocities. The assays were performed at 37 °C for 1 h with concentrations of L-ornithine from 100 μM to 10 mM. Optimal pH was determined by incubating enzyme solution with buffers at various pH values. (B) The K_m , V_{max} and k_{cat} values were determined with L-lysine as the substrate as for (A), except that the concentrations of L-lysine were varied from 500 μM to 10 mM. Values are the means $\pm$ S.E.M. for three or four experiments. NRO, no reaction observed in the substrate concentrations examined, thereby preventing kinetic studies from being performed.

(A)

Enzyme	K_m (μM)	V_{max} (nmol/min per mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Optimal pH
WT	562 $\pm$ 61	12.54 $\pm$ 1.0	77.78 $\pm$ 6.5	1.5 $\times 10^5$	8.0
K95A	17520 $\pm$ 2600	9.97 $\pm$ 0.4	0.74 $\pm$ 0.02	419.95	8.2
C96A	812 $\pm$ 103	11.22 $\pm$ 0.5	32.73 $\pm$ 1.7	4.1 $\times 10^4$	8.6
C338A	4559 $\pm$ 240	16.22 $\pm$ 0.5	10.54 $\pm$ 0.5	1507.62	8.0
C377A	398 $\pm$ 67	1.76 $\pm$ 0.04	3.37 $\pm$ 0.1	847.09	8.6

(B)

Enzyme	K_m (μM)	V_{max} (nmol/min per mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	Optimal pH
WT	1592 $\pm$ 120	0.152 $\pm$ 0.01	0.236 $\pm$ 0.1	148.00	6.8
K95A	4983 $\pm$ 870	0.054 $\pm$ 0.004	0.083 $\pm$ 0.03	75.96	7.2
C96A	1094 $\pm$ 40	0.133 $\pm$ 0.08	0.207 $\pm$ 0.1	188.83	6.8
C338A	1114 $\pm$ 130	0.150 $\pm$ 0.01	0.204 $\pm$ 0.1	183.21	7.6
C377A	NRO	NRO	NRO	NRO	7.4

Table 3 Mixed substrate experiments of ODC on L-ornithine and L-lysine

$V_{\max}$ values were determined from double-reciprocal plots for L-ornithine, L-lysine or mixture of L-ornithine and L-lysine. In the present experiments, substrates were used at different concentrations to ensure reasonably comparable velocities; the concentrations were approximately related to the Michaelis constants. In the mixed-substrate studies, 400 μ M L-ornithine and 1.2 mM L-lysine were used, showing their relative concentrations of 0.7 and 0.753, respectively. Values shown are the means $\pm$ S.E.M. for three or four experiments.

Substrate	$V_{\max}$ (nmol/min per mg)
L-Ornithine	12.54 $\pm$ 1.0
L-Lysine	0.152 $\pm$ 0.01
L-Ornithine + L-lysine	3.596 $\pm$ 0.354

Figure 4 Inactivation of ODC (●) and LDC (○) activity by DTNB

Purified enzyme was incubated with 1 μ M DTNB at 25 °C for the times indicated. After incubation, aliquots (1 μ g) were removed and assayed for the residual activity at 37 °C for 1 h. Inactivation of both ODC and LDC activities were detected in the enzyme. Results are from one representative experiment repeated with three enzyme preparations, and are expressed as the percentage of control enzyme activity.

L-lysine (Table 2). The K_m value for ODC was shown to be a 'true' dissociation constant [40]. A K_m value obtained for L-lysine that was only 3-fold higher suggested that ODC was unable to discriminate entirely between L-ornithine and L-lysine. This might imply that Schiff-base formation is relatively tolerant of variable side chains at the active site of ODC. However, as the decarboxylation reaction needs a fixed quinonoid intermediate, the length of the side chain is very important in forming the correct C(α)-N configuration. The 330-fold higher k_{cat} for L-ornithine compared with that for L-lysine is consistent with this interpretation. These studies show that *N. glutinosa* decarboxylase is a single enzyme exhibiting substrate specificity for both L-ornithine and L-lysine.

Chemical modification of ODC

In our previous reports, 1 μ M DTNB and 0.1 mM salicylaldehyde completely inhibited ODC activity, suggesting the involvement of lysine and cysteine residues in the active site (results not shown). DTNB was used further to determine whether cysteine residues were important in both ODC and LDC activity (Figure 4). DTNB reacts specifically and stoichiometrically with cys-

teine, and the extent of modification can be determined spectrophotometrically by monitoring the increase in absorbance at 412 nm [23]. The difference spectra recorded at various time intervals during the modification of the enzyme preparation confirmed that cysteine residues are being modified. Concomitantly with spectral changes, modification of cysteine residues by DTNB prevented both the ODC and LDC activities. In addition, the decrease in activity was dependent on DTNB concentration and incubation time (results not shown). These results suggest that the cysteine residues being modified by DTNB are vital for both ODC and LDC activity. As depicted in Figure 4, the LDC and ODC activity had different rates of inactivation by DTNB. These differences could be due to the low activity of LDC. Even though LDC activity was inhibited by DTNB, as a consequence of the low initial activity, only a small decrease in LDC activity was observed relative to the decrease in ODC activity. However, it is also possible that the active site of LDC is less accessible to DTNB, when the larger substrate L-lysine is bound instead of L-ornithine.

Sequence alignment and X-ray structure of ODCs from several eukaryotic sources [6–8,41] revealed that, among several important amino acids, Lys⁶⁹ and Cys³⁶⁰ are critical for catalytic activity. In order to perform site-directed mutagenesis, three conserved cysteine residues (Cys⁹⁶, Cys³³⁸ and Cys³⁷⁷) and one lysine residue (Lys⁹⁵) were selected for mutation by an examination of sequence that was conserved, and on the basis of the results of chemical modification experiments.

Kinetic analysis of wild-type and mutant ODCs

Results from the kinetic analyses of wild-type and four ODC mutant proteins are summarized in Table 2. Substrate specificity for the basic amino acids, L-ornithine, L-lysine and L-arginine, was measured for wild-type ODC. Since no activity was detected with L-arginine, kinetic analyses were performed only for L-ornithine and L-lysine (Table 2). Double-reciprocal plots were linear across the concentration range of both L-ornithine and L-lysine. The K95A mutant displayed the lowest ODC activity among the mutants, showing a 100-fold decrease in k_{cat} values and a 30-fold increase in K_m values. In terms of LDC activity, k_{cat} was reduced by 3-fold, with a greater-than-3-fold increase in K_m values compared with the wild-type enzyme. The highest increase in K_m values for both L-ornithine and L-lysine with the K95A mutant indicates that Lys⁹⁵ is an essential substrate-binding determinant in ODC. Mutation of Lys⁹⁵ to alanine decreased the k_{cat} value for L-ornithine by 100-fold, and for L-lysine by 3-fold. This can be explained by decreased rates of formation of the enzyme-substrate-Schiff-base complex and of decarboxylation in the mutant enzyme, since the K95A mutant would form an enzyme-bound PLP-aldehyde adduct instead of the enzyme-bound PLP-Schiff base formed by the wild-type enzyme. Lysine mutants totally lacking enzyme activity were also reported in alanine racemase from *Bacillus stearothermophilus* [42], bacterial histidine decarboxylase [43] and a number of transaminases [44–46]. This suggests that in plants too this lysine residue of ODC plays an essential role in the catalytic mechanism.

C96A had minimal effects on ODC activity, with a 2.3-fold decrease in k_{cat} and a 1.4-fold increase in the K_m for L-ornithine. The k_{cat} and K_m parameters for decarboxylating L-lysine were similar to those of wild-type enzyme. Although Cys⁹⁶ is positioned next to the essential lysine residue, mutation to alanine did not greatly affect the ODC activity. This result is consistent with a report describing a mouse mutant ODC, having Cys⁷⁰ altered to serine, also showing a 2-fold higher K_m value and a 2-fold lower k_{cat} [47]. The finding that the Cys⁹⁶ is not essential for the ODC

Figure 5 Inhibition of ODC by α -DFMO as a function of L-ornithine concentration

Purified enzyme (1 μ g) was incubated by varying the concentrations of ornithine from 200 μ M to 1.5 mM. The K_i value was examined with various concentration of L-ornithine in the absence (●) or presence of α -DFMO at a concentration of 0.5 μ M (○) or 1 μ M (▼). The calculated K_i for α -DFMO with ODC was 1.15 μ M. The values are means $\pm$ S.E.M. for three experiments.

activity is supported further by the amino acid sequence of *L. donovani*, which has a serine residue at the equivalent position [48]. The C338A mutant possessed 8-fold-higher K_m values, and k_{cat} values were 7-fold lower than for the wild-type. However, in terms of LDC activity, the K_m and V_{max} values were similar to those of the wild-type enzyme. The C377A mutant had very little residual ODC activity, in addition to a complete loss of LDC activity. The specificity constant was reduced by 23-fold for L-ornithine, despite a 1.4-fold decrease in the K_m , and this result was consistent with the decrease in k_{cat} by 26-fold despite a 2-fold decrease in the K_m for mouse ODC [47]. The k_{cat}/K_m value was reduced approx. 180-fold over that of the wild-type enzyme, and the V_{max} values were lowest among the mutant proteins. Moreover, the C377A mutant did not show sufficient activity towards L-lysine to enable kinetic studies to be performed. This suggests that Cys³⁷⁷ is critically involved in the active site.

Cys³⁶⁰ (C377A of *N. glutinosa* ODC) is also known to be important in the covalent binding site of α -DFMO [47]. α -DFMO inhibited ODC activity in a dose-dependent manner (Figure 5). Inhibition patterns were determined from double-reciprocal plots by using fixed α -DFMO concentrations and varying the L-ornithine concentrations. The K_i value of wild-type ODC for α -DFMO was 1.15 μ M, showing a competitive inhibition pattern. ODC isolated from mouse and *Panagrellus redivivus* had similar K_i values for α -DFMO of 39 and 34 μ M respectively [49,50], and K_i for *T. brucei* was calculated to be 220 μ M [51]. The fact that the K_i value for α -DFMO of *N. glutinosa* ODC was calculated to be the lowest shows that α -DFMO is a strong competitive inhibitor of plant ODC.

Optimal pH and thermal stability of wild-type and mutant ODCs

The mutant ODCs showed an altered optimal pH for ODC activity (Table 2A) from pH 8.0 (wild-type) to 8.6 (C377A, C96A), and from pH 6.8 (wild-type) to 7.6 (C338A) in terms of LDC activity (Table 2B). The optimal temperature for wild-type and mutant ODCs was 37 °C. The thermal stability of wild-type and mutant ODCs was measured by incubating PBS-diluted

Table 4 Half-life ($t_{1/2}$) for the thermal inactivation of wild-type and mutant ODCs at 40, 50 and 60 °C

Enzyme was present at a concentration of 1 mg/ml with PBS. Samples were incubated at 40, 50 and 60 °C. At various times, aliquots (1 μ g) were removed and assayed for residual ODC activity at 37 °C for 1 h. ' $t_{1/2}$ ' represents the loss of activity as a function of time (min). Values are means $\pm$ S.E.M. for three experiments.

Mutants	Temperature (°C) ...	$t_{1/2}$ (min)		
		40	50	60
WT		69.51 $\pm$ 5.7	20.45 $\pm$ 3.5	5.42 $\pm$ 0.8
K95A		16.27 $\pm$ 0.01	9.67 $\pm$ 0.6	3.01 $\pm$ 1.1
C96A		62.56 $\pm$ 5.1	13.79 $\pm$ 0.07	4.76 $\pm$ 1.1
C338A		13.36 $\pm$ 0.6	5.83 $\pm$ 0.2	5.02 $\pm$ 0.7
C377A		14.28 $\pm$ 0.5	5.96 $\pm$ 0.7	1.02 $\pm$ 1.0

Table 5 Secondary-structure content of wild type and mutant ODC

CD spectra were generated between 260 and 200 nm. The data was converted into molar ellipticity units and used in a Jasco secondary-structure estimation program [26] to determine the percentage of α -helix, β -sheet, turn and random structure. Each reported spectrum is the average of five measurements. Fractions of each structure were calculated by deconvoluting the observed spectra with the CD spectra.

Protein	Secondary structure element (% of overall content)			
	α -Helix	β -Sheet	Turn	Random
Wild-type	29.0	0.0	35.3	35.6
K95A	29.0	4.4	32.3	34.3
C96A	28.5	0.0	35.3	36.2
C338A	28.9	3.5	32.1	35.5
C377A	28.1	0.0	34.1	37.8

enzymes at 40°, 50° and 60 °C (Table 4). The pattern of thermal inactivation of wild-type and mutant ODCs obeys a pseudo-first-order reaction. The half-life ($t_{1/2}$) of thermal inactivation of the C96A mutant was similar to that of the wild-type ODC, but the $t_{1/2}$ measurements for K95A, C338A and C377A were very short. Adding PLP to the enzyme mixture was important in maintaining the thermal stability of the ODC protein. There was no decrease in enzyme activity detected for more than 2 h at 37 °C in the presence of PLP in the enzyme mixture. Similar results were reported in *S. ruminantium* LDC, where 50 % of the enzyme activity remained after incubating at 50 °C for 30 min when the enzyme was incubated with the cofactor PLP [17]. However, when the enzyme was incubated in the absence of PLP, half of the activity was lost after 30 min, even at 35 °C. Therefore a $t_{1/2}$ of 70 min or shorter in wild-type and mutant ODCs at temperatures > 40 °C demonstrates that the cofactor PLP is crucially needed to increase the thermal stability of *N. glutinosa* ODC. Analysis of the secondary structures of wild-type and mutant proteins by CD spectroscopy (Table 5) indicates that they were essentially the same, demonstrating that site-directed mutagenesis did not significantly alter the protein folding.

Secondary and tertiary structures of ODC

The secondary structure of *N. glutinosa* ODC was predicted by PSIPred (various protein structural-prediction methods at the University of Warwick; [27]), and is illustrated by the letters 'H' (α -helix) and 'B' (β -sheet) above the sequences in Figure 1. *N. glutinosa* ODC is predicted to have nine α -helices with lengths

Figure 6 The overall and active-site structure of *N. glutinosa* ODC generated by molecular modelling

(A) Overall three-dimensional structure of *N. glutinosa* ODC shown as ribbon diagrams. The α -helices and β -strands are indicated in red and blue respectively. (B) Enlarged image of ODC and mutated residues (Lys⁹⁵, Cys⁹⁶, Cys³³⁸ and Cys³⁷⁷) are drawn in ball-and-stick model representation. These Figures are drawn by SWISS-MODEL, an Automated Comparative Protein Modeling Server.

of 9–20 residues and 16 β -sheets with lengths of 4–13 residues in each monomer. The tertiary structure of *N. glutinosa* ODC was solved by superimposition of the structurally homologous mouse [6] *T. brucei* [7] and human [8] ODCs (Figure 6). The ODC structures were very similar, not only in terms of the packing of the molecules in the unit cell, but also in terms of details of the structure. The symmetrical homodimer of *N. glutinosa* ODC is formed by a head-to-tail interaction between the barrel of one domain and the sheet domain of the other (Figure 6). The α/β barrel from residues 66–288 forms the barrel domain, and the rest of the residues form the β -structure of the sheet domain. The barrel domain begins and ends with a helix (H2–H10), as found for the mouse ODC barrels [6], but different from barrels of triose phosphate isomerase (TIM), which usually starts with a β structure [52]. The C-terminal domain of the β -structure was six-stranded, having a single crossover connection, with additional parallel B-1 and B-18 strands (Figures 1 and 6). The C-terminal end of the α/β barrel possessed the binding site for cofactor PLP, mediated via a Schiff-base interaction, which is similar among other proteins utilizing this fold. The active site of ODC was formed in the superficial cavity formed at the interface of two subunits. The amino acid residues Lys⁹⁵ and Cys⁹⁶ were at the central cavity of the active site, and Cys³³⁸ was a residue of the loop involved in active-site formation. The functionally important Cys³⁷⁷ residue in ODC (Cys³⁶⁰ in *T. brucei* ODC) was found at the tip of the hairpin that was formed between two β -strands, B-14 and B-15 (Figures 1 and 6). The side chain of Cys³⁷⁷ was rotated away from the active site, directing the thiol group away from a position at which it could serve a catalytic role in the reaction. Cys³⁷⁷ might play a role in determining the configuration of the C(α)–N bond of the substrate, which would in turn dictate the position of the carboxy group relative to the plane of the PLP ring. Improper positioning of the carboxy group would result in a decrease in the decarboxylation rate. For the mutant C377A, the k_{cat} for L-ornithine was decreased by 23-fold, and no activity was detected for L-lysine, supporting this prediction. A 50-fold decrease in the k_{cat} for both *T. brucei* and mouse ODCs was also reported after mutation of this residue [47,53]. The Cys³³⁸ residue facing the active site was followed by a short helix of five

residues, which were highly conserved among other eukaryotic ODCs. The 8-fold increase in the K_m value for L-ornithine, and the decrease in the k_{cat} by 7-fold, suggest that this residue is involved in substrate binding.

This is the first report on the identification of the functionally important regions and residues in *N. glutinosa* ODC, having both LDC and ODC activities as determined by site-directed mutagenesis. By using comparative molecular modelling, the catalytic fold was predicted and presented herein as the first molecular model for plant ODC. Even though plants have an additional ADC pathway for putrescine biosynthesis, *N. glutinosa* ODC was stringent in terms of conserving a structure similar to that of other eukaryotic ODCs. In addition to the molecular modelling, X-ray crystallographic analysis of *N. glutinosa* ODC will provide further insights into the structure–function relationships of this enzyme, thus providing a basis for the design of specific regulators of polyamine biosynthesis in plants.

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