Ornithine Cyclodeaminase from Octopine Ti Plasmid Ach5: Identification, DNA Sequence, Enzyme Properties, and Comparison with Gene and Enzyme from Nopaline Ti Plasmid C58

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Octopine and nopaline are two arginine-derived opines synthesized in plant cells transformed with octopine or nopaline plasmids. Utilization in Agrobacterium tumefaciens is mediated by Ti plasmid regions called oec or noc (octopine or nopaline catabolism), and recent experiments showed that noc in pTiC58 codes for a pathway from nopaline to L-proline. The last enzyme is ornithine cyclodeaminase (OCD), an unusual protein converting L-ornithine directly into L-proline. We investigated whether octopine plasmid pTiAch5 also harbors a gene for OCD. The results revealed an ocd gene which is induced by octopine and maps in the oec region. Northern analysis and comparison with the gene from pTiC58 showed that the two genes are related (69% homology). The enzyme activity was characterized, and a comparison with OCD(C58) showed that the properties are similar, but not identical. Differences were detected in the regulation of enzyme activity by L-arginine and L-proline and in the response to varying ratios of NAD+/NADH. It is proposed that this reflects different mechanisms for integration of opine catabolism into general metabolism.

Ornithine cyclodeaminases (EC 4.3.1.12; OCDs) deaminate L-ornithine and convert the product directly into L-proline. This type of enzyme reaction has been demonstrated so far only in some anaerobic bacteria (20, 22), in two Pseudomonas strains (31), and in Agrobacterium tumefaciens, which may contain several of these genes (6, 11, 25). So far, only one has been investigated in detail [ocd(C58); 25]. It is part of the nopaline catabolism (noc) operon in nopaline plasmid pTiC58 (27), and the enzyme catalyzes the last step in a pathway leading from nopaline through L-arginine and L-ornithine to L-proline (Fig. 1).

Figure 1 also summarizes the question we are addressing. Octopine [N\(^2\)-(1-d-carboxyethyl)-L-arginine] is an opine which is structurally related to nopaline [N\(^2\)-(1,3-dicarboxypropyl)-L-arginine]: the carbonyl components are different (pyruvate or 2-oxoglutarate, respectively), but both contain L-arginine as amino acid. Genetic evidence indicated that L-ornithine is involved as an intermediate in the catabolism of both opines (5, 9, 14, 17, 27, 28). Nopaline and octopine plasmids are related, and therefore it would seem likely that octopine catabolism (ocd) utilizes the same reaction with L-ornithine as noc. Previous hybridization studies, however, failed to detect DNA homology between noc and ocd regions (10). On the other hand, recent experiments demonstrated that octopine plasmids do code for OCD activity (6, 11), and although the precise location of the gene was not identified, induction by octopine suggested that the gene is part of the ocd operon. The enzyme was found to be very unstable, and a preliminary characterization (6) revealed properties which were markedly different from those described for OCD(C58) (25).

Taken together, this seemed to suggest that both octopine and nopaline Ti plasmids contain ocd genes, but that they are diverged to such an extent that the DNA sequences do not cross-hybridize and the enzyme properties are different. This is of interest, and since OCDs are unusual and poorly characterized enzymes, we investigated the ocd gene in octopine plasmid pTiAch5 and analyzed the properties of the enzyme.

The results indicate that pTiAch5 contains an ocd gene which is part of the ocd region. It is closely related to ocd(C58); DNA as well as deduced amino acid sequences indicate about 69% identity. The enzymes have many properties in common, but there are also significant differences in the regulation of enzyme activity. These differences are discussed as part of the mechanisms which integrate opine utilization with general metabolism.

MATERIALS AND METHODS

Plasmids and bacterial strains. pTiB653 and pTiAch5 are two very closely related octopine wild-type plasmids (5, 7). pGV2260 is a derivative of pTiB653 in which the complete T region and some adjoining sequences are replaced by pBR322 (4), and pGV3850 is a derivative of nopaline Ti plasmid pTiC58 in which part of the T region is deleted and replaced by pBR322 (35). Agrobacterium sp. strain APF2 free of plasmids has been described (15). A. tumefaciens was routinely kept on agar plates with YEB medium (34) at 28°C. Escherichia coli plasmid pGV0230 harbors fragment HindIII of octopine plasmid pTiAch5 cloned in pBR322 (7). pGS227 and pGS251 are subclones from the noc region of pTiC58 (26); the latter expresses OCD(C58) as the sole noc region protein in E. coli.

DNA isolation from agrobacteria and hybridization. Total DNA was isolated, digested, and blotted to nitrocellulose by published procedures (8, 21). The hybridization probe from ocd(C58) was a HindIII-BclI fragment of 863 base pairs (25), which covers the complete protein-coding region except 140 base pairs at the 5' end. The isolated fragment was labeled by nick translation (kit from Bethesda Research Laboratories). Hybridizations were performed with 50% formamide under moderate stringency (21) for 12 to 14 h at 42°C; this

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was followed by three to five 30-min washes at 42°C with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium dodecyl sulfate.

Identification of ocd-encoded proteins in E. coli and Agrobacterium spp. The E. coli minicell system with strain DS410, analysis of the proteins labeled with L-[35S]methionine (37 TBq/nmol; Amersham Buchler) by sodium dodecyl sulfate-gel electrophoresis, and immunoprecipitations in E. coli extracts have been described before (3, 29). For identification of ocd gene products in Agrobacterium spp., cells grown at 28°C in liquid YEB medium (34) to about 1.5 A600 were pelleted by centrifugation and suspended in half of the original volume in induction medium (20 mM sodium citrate, pH 5.5, 2% sucrose) without or with opines (1 mM), or with other additions (see legend to Fig. 3). Cells were harvested after 3.5 h at 28°C. Proteins extracted with 80 mM Tris hydrochloride (pH 6.8) containing 2% sodium dodecyl sulfate were analyzed by the Western blotting (immunoblotting) technique. Polypeptides separated by gel electrophoresis (29) were transferred electrophoretically to sheets of nitrocellulose (33), and free protein attachment sites were blocked by treatment with Tween 20 (Sigma Chemical Co.) (1). Procedures for sequential incubation of the filters with rabbit antiserum and goat anti-rabbit antibodies coupled to alkaline phosphatase (Sigma) and the color reaction with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) have been published (18). Antiserum against OCD(C58) has been described previously (25).

OCD incubations. Enzyme extracts were prepared as described before (25) from E. coli DS410 harboring pNS10.1 [OCD(Ach3); see Results] or pGS251 [OCD(C58)] (25). They were dialyzed three times for 1 h each time against 20 mM potassium phosphate (pH 8.5) containing 50 mM NaCl and 10 μM NAD+. Incubations were routinely carried out in duplicate. Optimized standard assays for OCD(Ach5) contained the following, in a final volume of 20 μl: 7.5 μg of protein, 0.5 mM NAD+, 0.1 mM unlabeled L-ornithine, 17 μM L-[1-14C]ornithine (2 GBq/mmol; Amersham Buchler), 25 mM potassium phosphate, pH 8.5; incubation was at 30°C for 15 min. Under these conditions, the rate of conversion from L-ornithine into L-proline was linear with time up to 25 min, and <40% of the substrate was converted into the product. The same conditions were used for incubations with OCD(C58), but the protein amounts were increased to 22 μg in the presence or 55 μg in the absence of L-arginine to compensate for the lower specific activity of OCD(C58) (see Fig. 6). Whenever the conversion rate into L-proline exceeded 40%, incubation times were shortened to stay within these limits. Reactions were stopped by mixing with 5 μl of 50% trichloroacetic acid. The evaluation of OCD activity by quantitative thin-layer chromatography has been described before (25). It should be noted that L-ornithine and L-proline were separately quantified. Recovery of radioactivity in both was >97% of the input under all conditions tested. This excluded that substrate depletion by other reactions with L-ornithine in the crude extracts (e.g., ornithine decarboxylase) affected the results for OCD activity.

DNA sequence analysis. The ocd gene region was sequenced by the dideoxy nucleotide chain termination technique (24). The pTZ18/pTZ19 system and helper phage M13K07 (all from Pharmacia, Molecular Biology Division) and the reverse sequencing primer (Boehringer) were used routinely. We used procedures that followed the recommendations of the manufacturers, with slight modifications. Suitable subclones were obtained either by cloning of subfragments with BamHI, SalI, EcoRI, and SmaI and their combinations in both orientations into pTZ18 and pTZ19 or by additional construction of overlapping exonuclease III deletion clones (13). Standard molecular techniques were used throughout this work (21). About 95% of the DNA sequence shown in Fig. 4 was obtained from both strands or from overlapping, independent deletion clones.

RESULTS

ocd-related sequences in Agrobacterium spp. We first investigated by hybridization experiments whether Agrobacterium spp. harboring octopine plasmids (pTiB653 and pGV2260) contain sequences homologous to ocd(C58); strains CS8Cl(pGV3850) and APF2 were included, since the first harbors ocd(C58) (= positive hybridization control) and the second contains no plasmids (= probe for homologous chromosomal sequences). Total DNA was digested with various enzymes (HindIII, EcoRI, and BamHI); the fragments were separated by agarose gel electrophoresis, blotted to nitrocellulose, and hybridized to a nick-translated probe containing most of the coding region of ocd(C58) (see Materials and Methods).

All four strains revealed fragments reacting with this probe. The hybridizing sequences in APF2 must be localized on the chromosome. A previous report suggested that some Agrobacterium strains contain chromosomally encoded OCD activity (6); thus, they may represent an ocd gene, but this was not investigated further. CS8Cl(pGV3850) revealed additional bands, and by the pattern of hybridizing fragments they were identified as the ocd gene in the noc region.

Additional bands were also observed with the two strains harboring octopine plasmids. They were identical for
VOL. 171, occ
continues in that plasmid pTiAch5. 
HindIII), and by other HindIII and (7).
fragments corresponding to Ti are the known together, the 16b, plus region (Fig. 7), to homologous and agrobacteria 3, OCD(C58); 1 L-ornithine; 2.

Further experiments with pTiB6S3 and pGV230 did not possess OCD activity, and minicell experiments revealed no proteins expressed from HindIII 5. Assuming that the complete coding region is in the fragment, this suggested that the promoter was either not active in the heterologous background or missing in HindIII 5. The fragment was therefore recloned in both orientations into pNIIA (23) to provide vector promoters reading into HindIII 5. Figure 3, lane 4, shows that pNS10.1 (transcription from right to left with respect to the restriction map shown in Fig. 2) now expressed proteins with apparent sizes of 55, 33 to 34, and 14 kilodaltons (kDa) and closely spaced bands of 40 to 42 kDa in addition to the polypeptides encoded in the ampicillin resistance gene (see lane 1, vector control). The opposite orientation only produced some weakly expressed polypeptides (pNS10.2, lane 3; not visible in this print). The 40- to 42-kDa proteins from pNS10.1 corresponded in size to OCD from nopaline plasmid pTiC58 (lane 2), and they reacted with antiserum against OCD(C58) (not shown), indicating that the two proteins share antigenic sites. Enzyme assays revealed that extracts from E. coli cells with pNS10.1 possessed OCD activity, while extracts from pNS10.2 were inactive. Deletion of the internal fragment Smal 13 (Fig. 2) eliminated the 40- to 42-kDa polypeptides and also OCD activity, indicating that at least part of the gene resides in the Smal fragment.

ocd(Ach5): protein expression and activity. E. coli cells harboring pGV230 did not possess OCD activity, and minicell experiments revealed no proteins expressed from HindIII 5. Assuming that the complete coding region is in the fragment, this suggested that the promoter was either not active in the heterologous background or missing in HindIII 5. The fragment was therefore recloned in both orientations into pNIIA (23) to provide vector promoters reading into HindIII 5. Figure 3, lane 4, shows that pNS10.1 (transcription from right to left with respect to the restriction map shown in Fig. 2) now expressed proteins with apparent sizes of 55, 33 to 34, and 14 kilodaltons (kDa) and closely spaced bands of 40 to 42 kDa in addition to the polypeptides encoded in the ampicillin resistance gene (see lane 1, vector control). The opposite orientation only produced some weakly expressed polypeptides (pNS10.2, lane 3; not visible in this print). The 40- to 42-kDa proteins from pNS10.1 corresponded in size to OCD from nopaline plasmid pTiC58 (lane 2), and they reacted with antiserum against OCD(C58) (not shown), indicating that the two proteins share antigenic sites. Enzyme assays revealed that extracts from E. coli cells with pNS10.1 possessed OCD activity, while extracts from pNS10.2 were inactive. Deletion of the internal fragment Smal 13 (Fig. 2) eliminated the 40- to 42-kDa polypeptides and also OCD activity, indicating that at least part of the gene resides in the Smal fragment.

ocd(Ach5) is part of the occ operon. The results discussed so far indicated that octopine plasmid pTiAch5 contains an ocd gene which is localized either in or close to the occ region. If it is expressed as part of the occ operon, it should be induced by octopine in Agrobacterium spp. containing octopine Ti plasmids, and this was investigated in Western-blot-like experiments with antiserum against OCD(C58).
FIG. 4. DNA sequence of ocd(Ach5) and alignment with ocd(C58) (from reference 25); only nucleotide differences are shown. Restriction sites correspond to those in Fig. 2. —SD—, Deduced ribosome binding site for translation in E. coli (Shine-Dalgarno); +1, first nucleotide of the deduced AUG start codons (underlined). The stop codons at the end of the open reading frames are underlined and in lowercase letters.
which cross-reacts with OCD(Ach5). The results revealed that all of the Agrobacterium strains used in these experiments contained polypeptides of about 55 kDa which reacted with the serum. Their presence in APF2 (Fig. 3, lanes 9 to 11) argues that they are encoded in the chromosome. Expression in APF2 was not influenced by addition of nopaline, L-arginine, or L-ornithine to the medium, and neither octopine nor nopaline had an effect in Ti-plasmid-containing strains (lanes 5 to 8). The function of these polypeptides remains unknown. Immuno-reactive proteins of the size of OCD (40 to 42 kDa) were detected only in Ti-plasmid-containing Agrobacterium spp. and then only in the presence of the opines which induce the catabolic operons: nopaline with C58Cl1(pGV3850) (lane 7) and octopine with C58Cl1(pGV2260) (lane 5). The result obtained with Ti plasmid pGV2260 shows that ocd(Ach5) is part of the oct operon.

**DNA sequence and comparison with ocd(C58).** Hybridizations indicated the presence of ocd-related sequences in fragment EcoRI 32b, and the protein expression experiments showed that part of the gene extends into fragment Smal 13. This region was subcloned and sequenced, and the part containing the coding region is shown in Fig. 4 (see figure legend for position of the gene in Fig. 2). In the direction predicted by the protein expression experiments (from right to left in Fig. 2), the sequences revealed a single large open reading frame. Close to the 5’ end (position 1 in Fig. 4), it contains an AUG preceded by a Shine-Dalgarno sequence as found in many genes in E. coli (19), and the predicted protein size is 39.5 kDa. A doublet AUG 52 base pairs downstream lacks a typical Shine-Dalgarno sequence, but its use might explain the weak expression of a 1.9-kDa smaller protein, as detected in E. coli and Agrobacterium spp. (Fig. 3). Although this was not investigated further, it seems most likely that the predominant protein starts with the first AUG in the open reading frame. Beginning with this start codon, the deduced coding region (position 1 to 1068) is easily aligned with that of ocd(C58) (Fig. 4), and a comparison indicates that the two genes are related. Identity in this part is 68.5%, and most of the differences are scattered throughout the coding region. Homology drops off to <30% in the noncoding regions (only part of the known sequences are shown in Fig. 4). The 5’ region revealed no obvious promoter-like sequences; this is consistent with the observation that expression in E. coli requires vector promoters and that in Agrobacterium spp. the oct region genes are expressed as operons from a distant promoter. Taken together, sequence analysis suggests that ocd(Ach5) codes for a polypeptide of 39.5 kDa as the predominant gene product, and this is in reasonable agreement with the apparent size of the predominant protein expressed in E. coli minicells and in Agrobacterium spp. (42 kDa; Fig. 3).

The deduced amino acid sequence and comparison with ODC(C58) are shown in Fig. 5. OCD(Ach5) is two amino acids longer than ODC(C58) at the carboxy-terminal end; otherwise the protein sequences are 69.8% identical. Many of the differences may be considered as conservative exchanges, but there are also a considerable number of differences in charged amino acids. The functional significance is not clear, but at least some of them must be responsible for the observed differences in regulation of enzyme activity (see below). Analysis of the sequences for anabolic and catabolic ornithine carbamoyltransferases from different organisms revealed a strongly conserved cysteine-containing region which is most likely involved in binding of the substrate L-ornithine (summarized in reference 16), but similar sequences were not discovered in either one of the two ODCs. Since both enzymes require NAD’ for full

![Image](http://jb.asm.org/)

**FIG. 5.** Deduced amino acid sequence of OCD(Ach5) (a) and alignment with OCD(C58) (b). In the latter, only differences are shown. In the line between the sequences, * denotes differences considered as conservative exchanges and † denotes differences in charged amino acids.
activity, we also looked for the characteristic amino acid sequences and secondary structures which are often present in enzymes interacting with NAD⁺ (2), but we detected no obvious candidate sequences in either OCD.

**Enzymatic properties of OCD(Ach5) and comparison with OCD(C58).** All experiments were performed with the enzymes expressed in *E. coli* DS410 and under the conditions of an optimized standard assay (see Materials and Methods). Like OCD(C58), OCD(Ach5) was a stable enzyme in dialyzed extracts; they could be stored at -20°C for several months without loss of activity. Both required NAD⁺ for optimal activity, and the \( K_m \) values were very similar (Table 1). Differences were observed, however, when the cofactor was added in reduced form; OCD(C58) activity was more sensitive to inhibition by NADH than OCD(Ach5) (Table 2), suggesting that the two OCDs react differently to changes in redox states.

Previous results had shown that OCD(C58) activity is regulated in a complex manner by L-arginine (25); therefore, this was also investigated with OCD(Ach5). Figure 6 shows that OCD(C58) required a 0.1 to 1 mM concentration of the amino acid for full activity. No such activation was detected with OCD(Ach5), and in contrast to OCD(C58), L-arginine had no significant effect on the apparent \( K_m \) for l-ornithine (Table 1). Higher concentrations inhibited both enzymes, but the threshold values were different: 0.5 mM L-arginine reduced activity of OCD(Ach5) by about 50%, while OCD(C58) had highest activity at this concentration, and inhibition was observed only with >2.5 mM L-arginine (Fig. 6).

Tables 1 and 2 also summarize other properties of the two enzymes. There were significant differences in pH and temperature optima, and the enzymes differed in their response to other amino acids, for example, L-lysine and L-proline, one of the products of the OCD reaction. Ammonia, the other product, did not affect activity of either enzyme in concentrations up to 10 mM.

**DISCUSSION**

This study demonstrates that octopine plasmid pTiAch5 contains an *ocd* gene, and localization as well as induction by octopine show that it is part of the operon responsible for catabolic utilization of octopine in *Agrobacterium* spp. These results extend preliminary findings with a different octopine plasmid (6, 11), and they indicate that *occ* and *noc* regions code for similar enzyme reactions in L-ornithine catabolism.

The *ocd* genes and their protein products are not identical, since comparison of DNA as well as deduced amino acid sequences revealed significant differences. In the latter, they are not restricted to exchanges which may be considered as conservative; there are also 47 (13%) differences in charged amino acids. This led us to question whether the two enzymes possess different properties; the results of the comparative enzyme studies indicate that this is the case. Among others, significant differences were observed in the effects of L-arginine (precursor in the pathway), L-proline (product of the OCD reaction), and the NAD⁺/NADH (cofactor) ratio. Although conclusions from in vitro experiments with the enzymes expressed in *E. coli* must be considered cautiously, it is tempting to speculate that the observed differences reflect at least some aspects of the situation in *Agrobacterium* spp. in vivo.

**TABLE 1. Enzymatic properties of OCD(Ach5) and OCD(C58)**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Temp optimum (°C)</th>
<th>pH optimum</th>
<th>Apparent ( K_m ) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCD(Ach5)</td>
<td>30-35</td>
<td>8.0-8.5</td>
<td>( \text{L-Ornithine} )</td>
</tr>
<tr>
<td>OCD(C58)</td>
<td>25-35 (38-42)</td>
<td>8.0-9.5 (8.5-9.0)</td>
<td>( \text{L-Ornithine} )</td>
</tr>
</tbody>
</table>

\( * \) Values in parentheses were determined in the presence of the activating amino acid L-arginine (1 mM).

**TABLE 2. Effects of additions on enzyme activity**

<table>
<thead>
<tr>
<th>Addition (mM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCD(Ach5)</td>
</tr>
<tr>
<td>Standard assay</td>
<td>100</td>
</tr>
<tr>
<td>+ L-Proline</td>
<td>74</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>+ NADH*</td>
<td>100</td>
</tr>
<tr>
<td>0.05</td>
<td>81</td>
</tr>
<tr>
<td>0.5</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>+ L-Lysine</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
</tr>
</tbody>
</table>

\( * \) NADH instead of NAD⁺.

**FIG. 6. Effects of added L-arginine on OCD activities.** Note logarithmic scale for the L-arginine concentrations. Enzyme activities are given in nanomoles of L-proline produced per hour per milligram of protein. The data for OCD(C58) were taken from reference 25.
The Ti-plasmid-encoded catabolic pathway from octopine or nopaline proceeds through L-arginine, L-ornithine, and L-proline. All are amino acids required for several biosynthetic reactions, and their steady-state concentrations must be regulated such that catabolic and biosynthetic steps are optimally integrated. One of the mechanisms is that the catabolic genes are activated only in the presence of the inducing opines. In addition, our results suggest that different mechanisms on the level of enzyme activity regulation are used with OCD(Ach5) and OCD(C58) to achieve this goal. One reason for this may be that octopine and nopaline are available to Agrobacterium spp. in different quantities. Nopaline is the most abundant opine in nopaline tumors, while lysopine and opines of the agrobine-manopopine type, not octopine, are the major opines in octopine tumors (32). One could argue, therefore, that the intensity of the metabolite flow from nopaline is potentially higher than from octopine and that the observed differences in regulation of OCD are part of the mechanisms which accommodate and integrate the metabolite flow with basic metabolism. Further investigations with the enzymes expressed in Agrobacterium spp. are necessary to test the hypothesis. This has not been possible so far, since the instability of OCD in extracts from these bacteria prevented a detailed characterization of the properties (6). It also would be of interest to see whether other enzymes in the catabolic pathway, e.g., opine oxidases or the arginine identified as a product of the noc region, are subject to similar types of enzyme activity regulation.

In summary, our results show that opine-derived L-ornithine is converted to l-proline and ammonia in A. tumefaciens containing octopine or nopaline Ti plasmids. The ocd genes in the two plasmids are related, but evolutionary divergence is suggested by differences in DNA and protein sequences and in catalytic properties. The most striking difference appears to be in the regulation of enzyme activity by l-arginine: it would be interesting to investigate, by mutation analysis, which part of the protein is responsible for this property.

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LITERATURE CITED

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