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Cloning, Nucleotide Sequencing, and Expression of an Opine Dehydrogenase Gene from *Arthrobacter* sp. Strain 1C

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The gene coding for opine dehydrogenase from *Arthrobacter* sp. strain 1C was cloned onto plasmid pBluescript KS(−), and the nucleotide sequence of the 1,077-bp open reading frame consisting of 359 codons was identified as the *odh* gene. Transformed *Escherichia coli* cells overproduced NAD^+-dependent opine dehydrogenase under control of the promoter of the *lac* gene on pBluescript KS(−).

We purified and characterized a new enzyme, NAD^+-dependent opine dehydrogenase {ODH; *N*-[1-D-(carboxyl)ethyl]-L-phenylalanine:NAD^+ oxidoreductase (L-phenylalanine forming) (EC 1.5.1.—)} from a soil isolate of *Arthrobacter* sp. strain 1C (3). The enzyme has a molecular weight of about 70,000 and consists of two identical subunits with a molecular weight of about 36,000 (each). The enzyme catalyzes a reversible oxidation-reduction reaction of opine-type secondary amine dicarboxylic acids. In the oxidative deamination reaction, the enzyme is active toward opines, such as *N*-[1-D-(carboxyl) ethyl]-L-methionine (methiopine) (10) and *N*-[1-D-(carboxyl) ethyl]-L-phenylalanine. In the reductive secondary amine-forming reactions with NADH as a cofactor, the enzyme utilizes hydrophobic L-amino acids, such as L-methionine, L-isoleucine, L-valine, L-phenylalanine, and L-leucine, etc., as amino donors and α-keto acids, such as pyruvate, oxaloacetate, glyoxylate, and α-ketobutyrate, as amino acceptors. As far as we know, the enzyme is the only bacterial enzyme catalyzing this type of reaction to form the secondary amine dicarboxylic acid (Fig. 1).

In this paper, we report the cloning, sequencing, and expression in *Escherichia coli* of the *odh* gene from *Arthrobacter* sp. strain 1C, done not only to study the structural relationship with other proteins but also to synthesize a wide variety of natural and synthetic opine-type compounds containing two chiral centers, from free L-amino acids and α-keto acids (2). Such a secondary amine dicarboxylic acid structure is found in angiotensin-converting enzyme inhibitors, such as Enalaprilat and Lysinopril (20), etc.

Bacterial strain and plasmid. *Arthrobacter* sp. strain 1C (SCRC-1C, TPU582) was cultivated under the conditions described previously, with a medium containing chemically synthesized growth substrate *N*-[1-D-(carboxyl)ethyl]-L-phenylalanine (3). *E. coli* XL1-Blue MRF′ and pBluescript SK(+), SK(−), KS(+), and KS(−) were purchased from Toyobo (Osaka, Japan). *E. coli* was cultivated in Luria broth (LB) medium with 100 μg of ampicillin per ml. Plasmids were purified by using a plasmid purification kit from Qiagen.

Preparation of oligonucleotide probe and hybridization. We attempted to clone the *odh* gene by using an oligonucleotide derived from the N-terminal and internal amino acid sequences of the purified ODH enzyme, as described previously (12). The enzyme was purified from the cell extract of *Arthrobacter* sp. strain 1C by ammonium sulfate fractionation, DEAE-Toyopearl and butyl-Toyopearl column chromatographies, and Mono-Q and phenyl-Superose HR5/5 column chromatographies by fast protein liquid chromatography (Pharmacia, Uppsala, Sweden) (3). Prior to determination of the NH_2-terminal amino acid sequence, the purified enzyme was passed through a TSK phenyl-5PW column (0.75 by 7.5 cm; Tosoh Corp., Tokyo, Japan) and fractionated with a linear gradient of 20 to 80% (vol/vol) acetonitrile containing 0.05% (vol/vol) trifluoroacetic acid. The amino acid sequence of the N-terminal region of purified ODH (about 300 μg) was analyzed with an automatic protein sequencer 6625 (MilliGen). On the basis of the determined N-terminal amino acid sequences (MIESK TYAVLGLGNGGHAFAAYLALKGQ), an oligonucleotide probe (5′-GGIAACGGIGGICACGCITTCGCIGCITACCTI GCICTIAAGGGICAG-3′) (I designates deoxyinosine), which corresponds to G-13 to Q-28, was synthesized with a cyclone plus DNA synthesizer (MilliGen).

The genomic DNA of *Arthrobacter* sp. strain 1C was digested with restriction endonucleases (*Bam*HI, *Cla*I, *Eco*RI, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, and *Sph*I), electrophoresed, and transferred by blotting to a positively charged nylon membrane (Boehringer, Mannheim, Germany) with a Pharmacia VacGene XL instrument. The blotted DNA was hybridized with the [γ-^{32}P]ATP-labelled oligonucleotide probe. Hybridizations were carried out at 30°C for the first screening and at 60°C for the second screening. Optimum conditions for washing of the blotted DNA were sought by raising the stringencies of SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) solutions and monitoring the hybridization by autoradiography of the blotted DNA (BAS-1000, Fuji Photo Film Co., Ltd., Tokyo, Japan). Specific positive signals were detected in *Bam*HI (6.6 kb)-, *Kpn*I (4.4 kb)-, and *Sac*I (6.6 kb)-digested DNAs after washing with 0.2× SSC at 45°C for 30 min.

Cloning and sequencing of *odh* gene. Genomic DNAs of *Arthrobacter* sp. strain 1C were digested with *Sac*I and fractionated on a sucrose density gradient (10 to 40%) in a Beckman L-70 ultracentrifuge at 100,000 × *g* for 24 h. The 6- to 7-kb DNA fragments generated by the restriction enzyme *Sac*I were purified and ligated with *Sac*I-digested and dephosphorylated pBluescript KS(−) by using T4 ligase. The ligated DNA was used to transform *E. coli* XL1-Blue MRF′ to construct a genomic library of *Arthrobacter* sp. strain 1C. The genomic library was screened by colony hybridization under the same conditions described above for genomic Southern hybridization. One clone, pODHSS1, carrying the 6.6-kb *Sac*I fragment was selected for further analysis. The 5′-terminal region of the coding sequence of the *odh* gene was localized by

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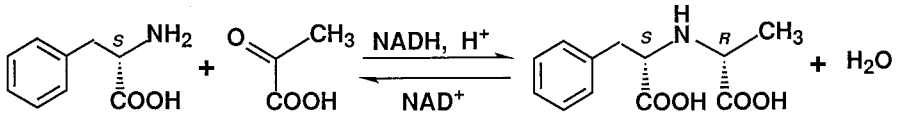

FIG. 1. Reaction catalyzed by ODH.

using a ^{32}P-end labelled oligonucleotide probe. The 2.1-kb *Pst*I-*Xba*I fragment, which was thought to contain the entire *odh* gene on the basis of the partially determined nucleotide sequence around the 5′-terminal region of the *odh* gene, was prepared from pODHSS1 and used for sequence analysis. The fragment to be sequenced was subcloned into pBluescript by standard procedures. To generate shorter clones suitable for sequencing, the exonuclease III deletion method for the kilo-sequencing kit (Takara Shuzo, Tokyo, Japan) was used. The sequence was determined by the dideoxy chain termination procedure of Sanger et al. (16), using α-^{35}S-dCTP. The nucleotide sequence of the 2.1-kb *Pst*I-*Xba*I fragment revealed an open reading frame, and translation of nucleotides +1 to +84 yielded an amino acid sequence identical to that obtained by degradative sequencing of the purified ODH. This open reading frame encodes a protein whose calculated molecular weight of 37,935 is in excellent agreement with the M_r of 36,000 estimated from the purified enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (3).

Expression of *odh* gene in *E. coli*. For expression of the *odh* gene in *E. coli*, pODH1 containing the 2.1-kb *Pst*I-*Xba*I fragment in pBluescript SK(−) was used. On the basis of the nucleotide sequence of pBluescript SK(−) and the 2.1-kb *Pst*I-*Xba*I fragment, the *odh* gene on pODH1 was expected to be expressed under control of the *lac* promoter on the vector. However, in the presence of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), *E. coli* harboring pODH1 did not grow in liquid culture, and the slow growth of colonies suggested that either the products of enzyme activity or expression of the enzyme itself might be toxic in *E. coli*. Moreover, the transformants did not exhibit any activities in the absence of IPTG. To overcome this problem, the transformant was cultivated at 37°C for 5 h in the absence of IPTG; thereafter, 1 mM IPTG (final concentration) was added to the culture, and incubation was continued for a further 7 h. The ODH activity was detected after 1 h, with a peak of expression after 5 h (1,090 U/liter of culture, 6.6-fold increase in activity relative to that of *Arthrobacter* sp. strain 1C) (3) followed by a gradual decrease in expression at later times. Analysis by SDS-PAGE (Fig. 2) revealed overproduction of a polypeptide with a molecular weight of about 37,000, that expected for ODH.

Deduced primary sequence and similarities to other proteins. The deduced primary structure of ODH was shown to be similar to a 40-kDa protein (17) (27.4% identity among 347 amino acids), D-lysopine dehydrogenase (EC 1.5.1.16, lysopine synthase) (6) (24.6% identity among 357 amino acids) (Fig. 3) and D-nopaline dehydrogenase (nopaline [octopine] synthase) (7) (20.8% identity among 168 amino acids) of *Agrobacterium tumefaciens*, D-nopaline dehydrogenase (15) of *Agrobacterium vitis* (21.8% identity among 371 amino acids), and phenylalanine dehydrogenase (1, 2, 14) of *Bacillus sphaericus* (21.8% identity among 78 amino acids). The genes encoding the first four proteins are all encoded by the Ti plasmid of *Agrobacterium* spp. The sizes of these proteins are almost the same as that of ODH, and some common residues are distributed within all four proteins. The similarities among these proteins are in the glycine-rich nucleotide binding domain G-11–X–G-13–X–X–(G or A)-16, connecting the β-strand with the α-helix in the region of ADP-binding βαβ folds, which is strongly conserved among NAD(P)$^+$-dependent dehydrogenases and FAD-containing oxidoreductases (4, 5, 18, 20, 22). However, phenylalanine dehydrogenase has one more domain important for accommodating the amino acid substrate located upstream (about 180 amino acids) of the ADP-binding βαβ fold. These opine dehydrogenases have the nucleotide binding domain that can be seen in the typical lactate dehydrogenases (11). There is also a similarity among these dehydrogenases and the 40-kDa protein, in the conserved D-238–X–X–R-241 residues, which are thought to be important for the proton relay mechanism (13, 20). It can be suggested that D-34 of ODH corresponds to D-214 of NAD$^+$-dependent phenylalanine dehydrogenase, the position of which is considered to be responsible for the binding of NADH discriminating NADPH among NAD(P)$^+$-dependent dehydrogenases (4, 9, 22). The positions equivalent to D-34 of ODH in the 40-kDa protein and D-lysopine dehydrogenase (octopine synthase, which utilizes both NADP$^+$ and NAD$^+$) are neutral S-30 and A-32, respectively, which are found in NADP$^+$-dependent dehydrogenases.

Although Schrell and Schröder (17) noted that the 40-kDa protein encoded between the *arc* (arginase) and *ocd* (ornithine cyclodeaminase) genes in the nopaline catabolic (*noc*) region shows no similarity with proteins other than D-isomer 2-hydroxyacid dehydrogenases, the similarity found in the overall part of these proteins strongly suggests that the 40-kDa protein could be both an NAD$^+$- and NADP$^+$-dependent dehydrogenase acting on an opine compound(s). There is also a similarity between ODH (residues 71 to 85) and glycerate dehydrogenase from cucumber (GDH) (17) as has been detected with the 40-kDa protein. There was little similarity with N^5-(L-1-carboxyethyl)-L-ornithine:NADP$^+$ oxidoreductase from *Streptococcus lactis*, which catalyzes the synthesis of N^5-(L-1-carboxyethyl)-L-ornithine and N^5-(L-1-carboxyethyl)-L-lysine by the reductive secondary amine-forming reaction with NADPH (20–22).

The physiological role of this enzyme may be to degrade

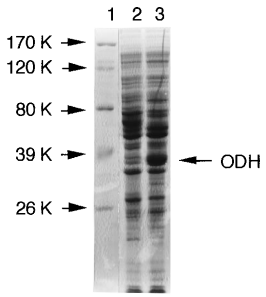

FIG. 2. SDS-PAGE of crude extract of *E. coli* transformant expressing the *odh* gene. The 2.1-kb *Pst*I-*Xba*I fragment was inserted into those sites of pBluescript KS(−) to construct pODH1. The overnight culture (300 ml) of *E. coli* XL1-Blue MRF′ cells harboring pODH1 was transferred into 30 ml of LB medium in a 300-ml Erlenmeyer flask and grown at 37°C for 5 h, IPTG (final concentration, 1 mM) was added to the culture, and incubation was continued for a further 7 h. A sample (1 ml) was removed at appropriate intervals, and the ODH activity was determined as described previously (3). The production of the ODH enzyme was also verified by SDS-PAGE. Lanes: 1, molecular mass markers, with sizes (in kilodaltons [K]) indicated on the left; 2, pBluescript KS(−); 3, pODH1.

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odh    1  MIESKTYAVLGLGNGGHAFAAYLALK-GQSVLAWD--IDAQRIKEIQDRGAIIAEGPGLA
                 *.** ** . ..*. ** . **    *.   . ........ *.   **. .
ldh    1    MAKVAILGAGNVALTLAGDLARRLGQVSSIWAPISNRNSFNSVRSLGSLELVGPDYG

odh   58  GTAHPDLLTSDIGLAVKDADVILIVVPAIHHASIAANIASYISEGQLIILNPGATGGALE
          *. .*. *..*.. *...*. *...**.. ...*  ..*..  .. ...  ** ....*.
ldh   58  GDFQPQ-LEDDLETAISGAAFIFLTVPTMGQQGILCELANFNLSSSVLVALPG-SATSLA

odh  118  FRKILRENGAPEVTIGETSSMLFTCRSERPGQVTVNAIKGAMDFACLPAAKAGWALEQIG
           ...*  . ** ... *...  ..**. . .** . ..* ... *. .* ... . . . .
ldh  116  CKQTLTPAFAP-IAVIEATTSPYACRRVN-AQVLMLSVKRTFEVASTQALSEE-VRGGFE

odh  178  SVLPQYVA-VENVLHTSLTNVNAVMHPLPTLLNAARCESG-TPFQYYLEGITPSVGSLAE
           ..*. ..  .*  . ..*.*.* **  .* . .  * * .*.. .   ..* . . ..
ldh  173  ILFPNRLQWYQNPASIFFSNTNPVAHPAGILAAKDTIEQGISPIPKFYRKFVPQAITRVT

odh  236  KVDAERIAIAKAFDLNVPSVCEWYKESYGQSPATIYEAVQGNPAYRGIAGPINLNTRYFF
           .*.**..*..*..*.  .  ...*. **  ...  * ..  .* .*..* *.* **.
ldh  233  AIDEERLTIVNALGLESETDFAYCKKWYGGHASNAREFYETFEGYADIETPRNMNHRYLS

odh  296  EDVSTGLVPLSELGRAVNVPTPLIDAVLDLISSLIDTDFRKEGRTLEKLGLSGLTAAGIR
          ***.  **   *.. ...*..* ...*..  *.....*....**.*..*.*.* .*..*
ldh  293  EDVKHILVLWVEIAEVIGVQVPEMKSVVQEASDVLNEDLSHTGRGLSSLNLEGSNANAIV

odh  356  SAVE
          .*..
ldh  353  RALNGV
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FIG. 3. Comparison of amino acid sequences of the *odh* gene product from *Arthrobacter* sp. strain 1C and D-lysopine dehydrogenase of *Agrobacterium tumefaciens*-infected crown gall. Computer alignments of the SWISS-PROT and NBRF-PIR database sequences were made with the DNASIS program (Hitachi, Tokyo, Japan). Of each pair, the first amino acid sequence (odh) is the sequence of the *odh* product from *Arthrobacter* sp. strain 1C (sequence size, 359 codons) (this study), and the second amino acid sequence (ldh) is that of D-lysopine dehydrogenase (lysopine synthase) of *Agrobacterium tumefaciens*-infected crown gall (sequence size, 358 codons). Symbols: *, identical residue; ·, similarity of functional group.

opines of plant origin to yield NADH rather than to synthesize the opines, as occurs in most cases with the NAD^+-dependent oxidoreductases. The products α-keto acid and L-amino acid could support the growth of the microorganism.

As far as we know, ODH is induced by a synthetic secondary amine dicarboxylic acid *N*-[1-D-(carboxyl)ethyl]-L-phenylalanine, whose preparation is rather laborious (3). Now that this enzyme has become available in quantity, it should be possible to synthesize a wide variety of natural and synthetic opines by using the overproduced ODH with an enzymatic regeneration of NADH.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession number D45211.

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