Purification and Cloning of a Proline 3-Hydroxylase, a Novel Enzyme Which Hydroxylates Free L-Proline to cis-3-Hydroxy-L-Proline

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Received 14 March 1997/Accepted 3 July 1997

Proline 3-hydroxylase was purified from Streptomyces sp. strain TH1, and its structural gene was cloned. The purified enzyme hydroxylated free L-proline to cis-3-hydroxy-L-proline and showed properties of a 2-oxoglutarate-dependent dioxygenase (H. Mori, T. Shibasaki, Y. Uosaki, K. Ochiai, and A. Ozaki, Appl. Environ. Microbiol. 62:1903–1907, 1996). The molecular mass of the purified enzyme was 35 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point of the enzyme was 4.3. The optimal pH and temperature were 7.0 and 35°C, respectively. The Km values were 0.56 and 0.11 mM for L-proline and 2-oxoglutarate, respectively. The kcat value of hydroxylation was 3.2 s⁻¹. Determined N-terminal and internal amino acid sequences of the purified protein were not found in the SwissProt protein database. A DNA fragment of 74 bp was amplified by PCR with degenerate primers based on the determined N-terminal amino acid sequence. With this fragment as a template, a digoxigenin-labeled N-terminal probe was synthesized by PCR. A 6.5-kbp chromosome fragment was cloned by colony hybridization with the labeled probe. The determined DNA sequence of the cloned fragment revealed a 870-bp open reading frame (ORF 3), encoding a protein of 290 amino acids with a calculated molecular weight of 33,158. No sequence homolog was found in EMBL, GenBank, and DDBJ databases. ORF 3 was expressed in Escherichia coli DH1. Recombinants showed hydroxylating activity five times higher than that of the original bacterium, Streptomyces sp. strain TH1. It was concluded that the ORF 3 encodes functional proline 3-hydroxylase.

Among eight stereoisomers of hydroxyprolines, only trans-4-hydroxy-L-proline is abundant in nature as a component of collagens produced by higher organisms (7). It is known that procollagen-proline dioxygenase (prolyl 4-hydroxylase; EC 1.14.11.2) hydroxylates L-proline residues of procollagen posttranslationally to trans-4-hydroxy-L-proline residues during collagen biosynthesis (7, 14, 21). Procollagen-proline 3-dioxygenase (prolyl 3-hydroxylase; EC 1.14.11.7), which hydroxylates peptide L-proline residues to trans-3-hydroxy-L-proline, is also involved in collagen biosynthesis (11, 17). However, free L-proline is not accepted as a substrate for these prolyl hydroxylases. These enzymes belong to a family of 2-oxoglutarate-dependent dioxygenases which require 2-oxoglutarate and O2 as cosubstrates and ferrous ion as a cofactor for the reaction. In contrast, hydroxylation of free L-proline to free cis-3-hydroxy-L-proline was reported that microbial proline 4-hydroxylase belongs to a family of 2-oxoglutarate-dependent dioxygenases as well as prolyl hydroxylases involved in collagen biosynthesis (9). Recently, Lawrence et al. have reported the ORF 3 encodes functional proline 3-hydroxylase. (13), as in the case of S. griseoviridus P8648 (9). However, details of the 3-hydroxylating activity remain to be investigated.

Here we report the purification and cloning of proline 3-hydroxylase from Streptomyces sp. strain TH1. This is the first report of the purified proline 3-hydroxylase and its structural gene.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise mentioned. cis-3-Hydroxy-L-proline was purified as described previously (13) and used as the standard in a high-performance liquid chromatography (HPLC) assay of this study.

Bacteria and culture conditions. Streptomyces sp. strain TH1 was isolated from soil from Tokyo, Japan (13). Frozen stock of TH1 cells was inoculated and reactivated in 200 ml of SR5 liquid medium (50 g of glucose, 10 g of soluble starch, 5 g of Bacto Yeast Extract [Difco Laboratories, Detroit, Mich.], 0.5 g of Bacto Tryptone [Difco], 3 g of meat extract, 0.5 g of MgSO4·7H2O·8H2O [pH 7.2], each per liter) at 28°C for 5 days. This activated seed culture was transferred to 3 liters of D3 medium (50 g of soluble starch, 30 g of corn steep liquor, 0.5 g of KH2PO4, 0.5 g of MgSO4·7H2O, 5 g of CaCO3, pH 7.0, each per liter) in a 5-liter jar fermentor, and TH1 was cultivated at 28°C for 1 day at an agitation rate of 500 rpm and harvested by centrifugation. One hundred grams of wet cells was obtained from 3 liters of TH1 liquid culture.

Enzyme assay. Proline 3-hydroxylase activity was assayed by measuring the formation of cis-3-hydroxy-L-proline from L-proline. The concentration of cis-3-hydroxy-L-proline in the mixtures was measured by use of the HPLC system with the postcolumn derivatization method with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole described previously (16) throughout this study. One unit of activity is defined as the conversion of 1 nmol of L-proline to 1 nmol of cis-3-hydroxy-L-proline per min.

During purification, proline 3-hydroxylase activity was assayed with 100 μl of the reaction mixture, which contained 100 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer (pH 7.5), 5 mM 2-oxoglutarate, 5 mM L-proline, 5 mM l-ascorbate, 1 mM FeSO4·7H2O, and 10% (vol/vol) enzyme fraction. Each reaction mixture was incubated at 30°C for 30 min.

After purification, properties of the purified enzyme were determined by use of the following reaction conditions unless otherwise mentioned: 100 mM TES buffer (pH 7.0), 5 mM 2-oxoglutarate, 5 mM L-proline, 5 mM l-ascorbate, 1 mM FeSO4·7H2O, and 0.17 U of purified enzyme in 100 μl of reaction mixture at 35°C. Under these conditions, cis-3-hydroxy-L-proline was produced linearly for up to 10 min. Kinetic parameters were calculated from velocity data of 5-min
FIG. 1. Physical structures of plasmids. All plasmids are illustrated in line diagrams without vector structure except for the MCS, which is located to the right of the inserted fragment. The sequenced region (2.9 kbp) shown in Fig. 5 is indicated at the top of the figure. Predicted ORFs are illustrated as open arrow-boxes. To construct pTH40, the 4.2-kbp EcoRI fragment indicated by a double-headed arrow was deleted as described in Materials and Methods. One of two used EcoRI sites was derived from the MCS of the vector as indicated in the figure. Although pTH40 has other KpnI and Smal sites, only those used to cut out ORF 3 are marked below the physical map of pTH40. The number under each restriction site indicates its position in Fig. 5. The EcoRV site in the MCS just downstream of the EcoRI site was used to cut out ORF 3 from pTH40 to construct pTH50 and is labeled EcoRV in the figure.

Purification of 3-hydroxylase. All operations were performed at 4°C. Column chromatographies were operated with a fast protein liquid chromatography system (Pharmacia). Thirty grams of frozen cells was suspended in 200 ml of buffer A (20 mM imidazole [pH 7.3], 1 mM diethyldithiocarbamate [DDT], 0.2 mM EDTA, 0.1% [vol/vol] Tween 20, 10% [vol/vol] glycerol) and disrupted by sonication. After removal of cell debris by centrifugation (30,000 × g, 30 min), the pH of the supernatant was adjusted to 4.3 with 6 N HCl. This acidified lysate was centrifuged (15,000 × g, 30 min) again, and the supernatant was loaded onto a RESOLUTE Q column (bed volume, 6 ml; Pharmacia) preequilibrated with buffer A. The sample-loaded column was washed with 70 ml of buffer A and eluted with a 120-ml linear gradient of 0 to 0.3 M NaCl in buffer A. Fractions of 6 ml were collected at a flow rate of 10 ml/min. The 3-hydroxylase activity was eluted at around 5 ml. Active fractions (18 ml) were pooled and diluted three times with buffer B (20 mM TES [pH 7.5], 1 mM DTT, 0.2 mM EDTA, 10% [vol/vol] glycerol) and then applied to a RESOURCE Q column (bed volume, 1 ml) preequilibrated with buffer B. The column was washed with 50 ml of buffer B and eluted with a 20-ml linear gradient of 0 to 0.3 M NaCl in buffer B. Fractions of 1 ml were collected at a flow rate of 10 ml/min. The 3-hydroxylase activity was eluted at around 6 ml. Sodium chloride was added to the pooled active fractions (7 ml) at a final concentration of 2 M. This solution was loaded onto a phenyl-Sepharose HP HiLoad column (bed volume, 1 ml; Pharmacia) preequilibrated with buffer B containing 2 M NaCl and eluted with 15 ml of buffer B containing 0.1% [vol/vol] Tween 20. Fractions of 1.25 ml were collected at a flow rate of 0.25 ml/min. The 3-hydroxylase activity was eluted in the first three fractions. The buffer of the pooled active fractions was replaced with buffer A with a PD10 column (Pharmacia), and then the active solution was loaded onto a RESOURCE Q column (bed volume, 1 ml) preequilibrated with buffer A. The sample-loaded column was washed with 10 ml of buffer A and eluted with a 20-ml linear gradient of 0 to 0.3 M NaCl in buffer A. Fractions of 1 ml were collected at a flow rate of 2 ml/min. The 3-hydroxylase activity was eluted at around 11 ml. Active fractions (2 ml) were pooled and diluted three times with buffer B containing 0.1% [vol/vol] Tween 20 and then loaded onto a RESOURCE Q column (bed volume, 1 ml) preequilibrated with buffer B containing 0.1% [vol/vol] Tween 20. After the column was washed with 10 ml of buffer B containing 0.1% [vol/vol] Tween 20, the enzyme was eluted with a 20-ml linear gradient of 0 to 0.3 M NaCl in buffer B containing 0.1% [vol/vol] Tween 20. Fractions of 1 ml were collected at a flow rate of 2 ml/min. The 3-hydroxylase activity was eluted in the 19th fraction. The protein concentration was assayed by the method of Lowry et al. (10).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed (8) on a 12.5% polyacrylamide gel (5 by 5 cm) and stained with Coomassie brilliant blue (CBB). An acrylamide gel of 11% (12 by 10 cm) was used for staining with silver. The isoelectric point of the purified enzyme was determined by isoelectric gel electrophoresis and CBB staining performed with the Phast-system (Pharmacia) as described in the manufacturer's instructions.

Molecular manipulation of genes. All DNA manipulations were performed as described by Sambrook et al. (19) except as otherwise mentioned. Escherichia coli XL-2 Blue MRF* (Stratagene, La Jolla, Calif.) was used as a host for gene cloning. PCR amplifications were performed with Pfu DNA polymerase (Boehringer Mannheim GmbH) under conditions prescribed by the supplier except for the addition of 10% (vol/vol) dimethyl sulfoxide to reaction mixtures. PCR products were analyzed by electrophoresis on 15% polyacrylamide gels. The DNA sequence was determined by the termination method (20) with a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Mass.) and a model 373A autosequencer (Applied Biosystems).

PCR-based cloning of the N-terminal portion of proline 3-hydroxylase structural gene. Several degenerate primers were synthesized on the basis of the determined N-terminal amino acid sequence, at positions 1 to 6 (MCSHIL) and 21 to 25 (EYLAT), for a sense and an antisense primers, respectively, to amplify a 74-bp N-terminal fragment of the proline 3-hydroxylase structural gene. This design of primers, the undetermined second amino acid was assumed to be cysteine for convenience. The second amino acid was later found to be arginine from DNA sequence analysis (see Fig. 5). All combinations of sense (10 μM) and antisense (10 μM) primers were tested for PCR amplification. Only the combination of sense primer A1 [5′-ATGGTT(C/T)CT(T/C/A)GA(C/T/T)AT(T/C/A)GTC(T/C/G)C(T/G)T-3′] and antisense primer B2 [5′-GT(T/C/A)G(C/T/G)TC(T/A)AG(A/G)TAT(T/G/C)TAC(T/G/C)-3′] amplified a 74-bp fragment. The fragment was recovered from a gel electrophoretically and cloned into pUC19 with SureClone (Pharmacia). The sequence of the cloned fragment was found to be identical to the determined N-terminal sequence of the purified protein except that the undetermined second residue was cysteine. By use of the cloned N-terminal fragment as a template, digoxigenin (DIG)-labeled N-terminal probe was synthesized by PCR with...
Molecular cloning of the entire structural gene. TH1 genomic DNA was digested with PstI and electrophoresed in a 1% agarose gel. A portion of the gel including 6.5-kbp DNA fragments was cut out, and DNA was recovered from the gel piece with a Prep-A-Gene kit (Bio-Rad Laboratories, Hercules, Calif.). Extracted DNA fragments were ligated into the PstI site of a vector plasmid, pBlueScript II KS + (Stratagene). Transformants were obtained on nylon membranes (HighBond N; Amersham) placed on Luria-Bertani plates containing 90 mg of ampicillin per liter. Colony hybridization was performed with the DIG-labeled N-terminal probe and DIG Detection Kit (Boehringer Mannheim GmbH). Extracted DNA fragments were ligated into the pBluescript II KS + vector and religated. The majority of transformants with the religated DNA had a plasmid which lacked the 4.2-kbp fragment. One of the deletion plasmids was selected and designated pTH40 (Fig. 1). In pTH40, the EcoRI fragment that exists downstream of the proline 3-hydroxylase gene was digested with EcoRI and religated. The majority of transformants with the religated DNA had a plasmid which lacked the 4.2-kbp EcoRI fragment. One of the deletion plasmids was selected and designated pTH40 (Fig. 1).

Construction of expression plasmids and E. coli recombinants. To delete a 4.2-kbp EcoRI fragment that exists downstream of the 3-hydroxylase gene, pTH40 was digested with EcoRI and religated. The majority of transformants with the religated DNA had a plasmid which lacked the 4.2-kbp EcoRI fragment. One of the deletion plasmids was selected and designated pTH40 (Fig. 1). In pTH40, the EcoRI site of a vector plasmid, pBluescript II KS + (Stratagene) digested with KpnI and Smal to locate the proline 3-hydroxylase gene downstream of lac promoter of the vector. E. coli DH1 (3) was used for expression of recombinant proline 3-hydroxylase.

Hydroxylation activity of E. coli recombinants. Recombinants were grown in 1.5 ml of Terrific Broth (19) supplemented with 50 mg of ampicillin per liter and 0.2 mM isopropyl-β-D-thiogalactopyranoside (medium A) at 30°C for 12 h, and a portion of the culture (15 μl) was inoculated into 1.5 ml of fresh medium A for further incubation at 30°C for 12 h. Cells of each recombinant were harvested by centrifugation from the 1.5-ml culture. Reaction mixtures (25 mM L-proline, 50 mM 2-oxoglutarate, 8 mM L-ascorbate, 4 mM FeSO4 ∙ 7H2O, 100 mM TES [pH 7.5], and cells from 1.5 ml of culture in 250 μl) were incubated at 35°C for 10 min. Reactions were stopped by heating at 100°C for 2 min and centrifuged. Supernatants were assayed by the HPLC method to quantify the concentration of cis-3-hydroxy-L-proline.

Nucleotide sequence accession number. The nucleotide sequence determined was registered in the DDBJ database under accession no. D78337.

RESULTS

Purification of proline 3-hydroxylase and its amino acid sequence. Streptomyces sp. strain TH1 was selected as a high producer of proline 3-hydroxylase from four microbial strains having 3-hydroxylase reported previously (13). After several culture media were tested, Dif medium was chosen for cultivation of TH1 to express 3-hydroxylating activity (data not shown). Cultured TH1 cells were disrupted by sonication in the buffer with pH 4.5 and centrifuged, since proline 3-hydroxylase activity of TH1 was not precipitated at this pH. The pH of the supernatant was readjusted to 4.5, and the supernatant was centrifuged again to precipitate neutral and basic proteins. The supernatant obtained was subjected to successive column chromatographies. Tween 20 was effective in achieving better separation of each column chromatography and was used throughout the purification procedure except for steps 4 and 5 as described in Table 1. Step 4 of the purification was performed without Tween 20 (Table 1) to eliminate it, since proline 3-hydroxylase activity was not adsorbed to a phenyl-Sepharose HP column used in the next chromatography step (Table 1, step 5) in the presence of Tween 20. Proline 3-hydroxylase was finally purified 265-fold from the cell lysate as shown in Table 1. The purified sample showed a single 35-kDa band in the SDS-PAGE gel stained with CBB (Fig. 2), although several minor bands were still detectable in the purified sample by silver staining (data not shown). Since proteins are stained with dyes in amounts proportional to their concentrations, but not when they are stained with silver (1, 22), it was concluded that 3-hydroxylase was purified to near homogeneity as a 35-kDa protein.

TABLE 1. Summary of purification of 3-hydroxylase from Streptomyces sp. strain TH1

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
<th>Tween 20 addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>542</td>
<td>3,540</td>
<td>6.5</td>
<td>1</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>106</td>
<td>1,800</td>
<td>17</td>
<td>3</td>
<td>51</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>7.5</td>
<td>1,553</td>
<td>207</td>
<td>32</td>
<td>44</td>
<td>+</td>
</tr>
<tr>
<td>4.</td>
<td>3.3</td>
<td>1,036</td>
<td>314</td>
<td>48</td>
<td>29</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>0.16</td>
<td>90.5</td>
<td>437</td>
<td>67</td>
<td>5.3</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>0.035</td>
<td>60.3</td>
<td>1,723</td>
<td>265</td>
<td>1.7</td>
<td>+</td>
</tr>
</tbody>
</table>

FIG. 2. SDS-PAGE of 3-hydroxylase. A purified sample (175 ng) was electrophoresed and stained with CBB. Lanes: 1, purified enzyme (Table 1, step 7); 2, markers of indicated molecular mass.
Further experiments are necessary to determine the native molecular mass of the 3-hydroxylase.

The isoelectric point of purified enzyme was determined to be 4.3 by isoelectric gel electrophoresis (see Materials and Methods). The optimal pH and temperature were 7.0 and 35°C, respectively. No cis-3-hydroxy-L-proline was detected in the reaction without L-proline and 2-oxoglutarate (Fig. 3C). Ferrous ion was strictly required for 3-hydroxylation of L-proline (Fig. 3B). The addition of EDTA (2 mM), a chelator of divalent cations, also inhibited the reaction strongly to less than 5% of the optimal. In a previous paper, crude cell extract of TH1 hydroxylated L-proline to some extent without the addition of ferrous ion (13). This discrepancy is thought to result from contaminated ferrous ion in the crude extract.

L-Ascorbate was not necessary to the reaction; however, acceleration of the reaction was observed with L-ascorbate (Fig. 3A). These results indicate that the purified proline 3-hydroxylase belonged to the 2-oxoglutarate-dependent dioxygenase family (2). Kinetic parameters were as follows. The $K_m$ for L-proline and 2-oxoglutarate were 0.56 and 0.11 mM, respectively. The $k_{cat}$ of cis-3-hydroxy-L-proline production was 3.2 s$^{-1}$ (Fig. 4).

The effects of metal salts and organic acids in the 3-hydroxylation reaction were tested. Of the metals tested, the addition of Co$^{2+}$, Cu$^{2+}$, or Zn$^{2+}$ at a concentration of 1 mM strongly inhibited the reaction to less than 10% of the optimal. Citric acid (5 mM) also inhibited the reaction to about 30% of the optimal. Succinate (5 mM), which is the end product of the 2-oxoglutarate-dependent dioxygenase reaction, had no effect on the reaction.

Substrate specificity of 3-hydroxylase. Instead of L-proline, proline analogs, i.e., D-proline, trans-4-hydroxy-L-proline, cis-4-hydroxy-L-proline, cis-4-hydroxy-D-proline, and 3,4-dehydroproline, were added to the 3-hydroxylation reaction mixture at a concentration of 5 mM. We could not detect a significant amount of any product with the HPLC system used (data not shown). To the extent tested, L-proline was thought to be the primary substrate for this 3-hydroxylase.

Cloning and sequencing of the proline 3-hydroxylase structural gene. The N-terminal portion of the structural gene was cloned by the PCR method and used as a template to synthesize DIG-labeled N-terminal probe. The DNA library of PstI-digested TH1 chromosome was screened by the colony hybridization method with the DIG-labeled N-terminal probe (see Materials and Methods). Two positive colonies were obtained, and each one was found to have a plasmid with the same physical structure. One of the plasmids was designated pTH30 (Fig. 1). The cloned fragment in pTH30 has one internal PstI site (Fig. 1). Since the larger PstI fragment within pTH30 contained the 3-hydroxylase gene and was shorter than the positive fragment resulting from Southern hybridization of PstI-digested TH1 chromosome (data not shown), it is assumed that the internal PstI site of the cloned 6.5-kbp fragment

![FIG. 3. Effect of various concentrations of cofactors. Reactions were performed as described in Materials and Methods except that the incubation time was 15 min. Accumulated amounts of cis-3-hydroxy-L-proline (3HYP) were plotted against concentrations of added cofactors. Concentrations of L-ascorbate, FeSO$_4$·7H$_2$O, and 2-oxoglutarate were varied in experiments A, B, and C, respectively.](http://jb.asm.org/file/5680/fig3.jpg)

![FIG. 4. Double-reciprocal plot of 3-hydroxylation at various concentrations of L-proline. A double-reciprocal plot between initial velocities of cis-3-hydroxy-L-proline accumulation (V) and concentrations of added L-proline (S) is shown. Kinetic parameters were obtained from the Michaelis-Menten equation fitted by least-squares regression analysis. The $K_m$ of L-proline was calculated to be 0.56 mM; the $k_{cat}$ was calculated to be 3.2 s$^{-1}$, assuming that the molecular mass of 3-hydroxylase was 35 kDa. The $K_m$ of 2-oxoglutarate was determined in the same way (Materials and Methods).](http://jb.asm.org/file/5680/fig4.jpg)
FIG. 5. Nucleotide sequence and predicted amino acid sequences of the cloned fragment from *Streptomyces* sp. strain TH1. The deduced amino acid sequences are shown just above the DNA sequences in a single-letter code, beginning with the first nucleotide of each codon. Predicted ORFs are surrounded by open boxes and designated as indicated to the right of the sequence. Shaded boxes represent the amino acid sequences identical to those determined of the purified protein.
TABLE 2. Proline 3-hydroxylating activity of recombinant strains with expression plasmids

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Inserted fragment</th>
<th>Amt of used cells (mg)</th>
<th>3HYP (nM)</th>
<th>Activity (U/mg of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTH45</td>
<td>Kpn1-Smal</td>
<td>29</td>
<td>0.52</td>
<td>0.57</td>
</tr>
<tr>
<td>pTH50</td>
<td>Kpn1-EcoRV</td>
<td>35</td>
<td>0.76</td>
<td>0.56</td>
</tr>
<tr>
<td>pBluescript II KS*</td>
<td>None</td>
<td>32</td>
<td>ND*</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND, not detected.

A 2.9-kbp portion of the cloned fragment was sequenced (Fig. 1). The determined sequence is shown in Fig. 5. One truncated open reading frame (ORF 1) and three complete ORFs (ORFs 2 to 4) were found to be arranged in the same direction. ORFs 2 to 4 encoded 303, 290, and 208 amino acids, respectively. Start codons of ORFs 2 and 3 were GTG. The GC content of the determined sequence was 67.5%. The N-terminal and internal amino acid sequences predicted by the determined DNA sequence of ORF 3 (Fig. 5) were identical to those of the purified protein. The undetermined second residue of the purified protein was revealed to be arginine. It was concluded that ORF 3 is the structural gene of the purified protein. The GC content of ORF 3 was 64.4%. Codons of ORF 3 have a G or C in position 3 at a frequency of 91%, which was as high as those of other genes of streptomycetes.

A homology search by BLAST in GenBank, EMBL, and DDBJ databases found no apparent homolog to ORFs 1 to 4. Subcloning and expression of proline 3-hydroxylase in E. coli. A 4.2-kbp EcoRI fragment was deleted from pTH30 to construct pTH40 (Fig. 1); ORF 3 was cut out of this plasmid with Kpn1-Smal or Kpn1-EcoRV. Cut-out DNA fragments were subcloned into an expression vector, pBluescript II KS*, digested with Kpn1-Smal, so that ORF 3 was located downstream of the lac promoter beyond the Kpn1 junction. E. coli DH1 was then transformed with constructed plasmids. Sequences of constructed plasmids were confirmed. Hydroxylating activities of transformants were measured in triplicate (see Materials and Methods). As shown in Table 2, recombinants with expression plasmids showed hydroxylating activity five times higher than that of the original Streptomyces sp. strain TH1. Both recombinant strains with expression plasmids had almost the same cellular activities. The different 3'-noncoding regions of two expression plasmids (Fig. 1) seemed to have no effect on the expression of ORF 3. It was concluded that ORF 3 encodes functional proline 3-hydroxylase.

DISCUSSION

We reported here the purification and cloning of proline 3-hydroxylase, which hydroxylates free L-proline to cis-3-hydroxy-L-proline. The purified enzyme strictly required ferrous ion and 2-oxoglutarate for its activity. On the other hand, L-ascorbic acid was not necessary for the hydroxylation, although it accelerated the reaction. This requisite profile of purified enzyme is similar to that of hyoscyamine 6β-hydroxylase (H6H) from henbane (4) but does not agree with that of prolyl 4-hydroxylase (EC 1.14.11.7), which strictly requires L-ascorbate (6, 21). H6H is a monomeric single-peptide enzyme of about 40 kDa and has apparent sequence homology with other monomeric 2-oxoglutarate-dependent dioxygenases (18) in spite of the fact that prolyl 4-hydroxylase is a heteromer consisting of 2α and 2β subunits (6). Although the native molecular mass of proline 3-hydroxylase was not determined, SDS-PAGE of the purified protein and expression experiments of ORF 3 clearly showed that a 35-kDa protein alone was sufficient for hydroxylating activity and no other heterocomponent was needed. Judging from properties described above, proline 3-hydroxylase probably belongs to a 2-oxo-acid-dependent dioxygenase subfamily composed of monomeric enzymes including H6H (2, 18). However, the deduced amino acid sequence of proline 3-hydroxylase showed no apparent homology to that of H6H and prolyl 4-hydroxylases from several origins. Further characterization is necessary to reveal the relationships between 3-hydroxylase and other 2-oxoglutarate-dependent dioxygenases.

Lawrence et al. reported the purification of proline 4-hydroxylase (9). This proline 4-hydroxylase is a single-peptide enzyme with a molecular mass of 38 kDa which has strict requirements for ferrous ion and 2-oxoglutarate. The reported Km value of l-proline is 0.45 mM. Both Co2+ and Zn2+ were strong inhibitors of the reaction. These reported characteristics of proline 4-hydroxylase resemble those of proline 3-hydroxylase in this study. However, these two hydroxylases are quite different in the following two points. First, L-ascorbate accelerated the 3-hydroxylation (Fig. 3A) but was reported to inhibit the 4-hydroxylation (9). Lawrence et al. proposed that l-ascorbate could compete with 2-oxoglutarate for the same binding site on 4-hydroxylase (9). However, in this study, excess L-ascorbate never inhibited the 3-hydroxylating reaction (Fig. 3A). Second, the specific activity of the purified proline 3-hydroxylase (1,723 nmol of cis-3-hydroxy-L-proline/min per mg of protein [Table 1]) was much higher than that of 4-hydroxylase (907 pmol/min per mg of protein [9]). These two hydroxylases, which are quite similar but critically different in a few features, are attractive materials for investigating relationships between structure and function.

Proline 3-hydroxylase is a rare enzyme in nature (13), and its specific activity is relatively weak as shown in this study. Fortunately, the structural gene of proline 3-hydroxylase of TH1 was capable of being expressed in the recombinant E. coli system (Table 2). However, the expressed 3-hydroxylase activity was still weak, as described in this study. Overexpressed 3-hydroxylase would be useful for further characterization of the 3-hydroxylase. We have already made several expression plasmids with higher activity. Detailed characteristics of the 3-hydroxylase will be revealed soon.

We have previously reported that cis-3-hydroxy-L-proline was detected in the hydrolysate of TH1 culture broth extract (13). It is probable that TH1 produced a compound including the cis-3-hydroxy-L-proline residue. Although further study is necessary to elucidate the physiological function of proline 3-hydroxylase, we assume that proline 3-hydroxylase plays a role in the biosynthesis of a peptide antibiotic as in the case of proline 4-hydroxylase in etamycin biosynthesis (5, 15).

Until now, only two hydroxylases of free l-proline have been reported. The first is proline 4-hydroxylase (9), and the second is the proline 3-hydroxylase described in this study. We suppose that there exist other types of free l-proline hydroxylases which produce cis-4-hydroxy-L-proline or trans-3-hydroxy-L-proline. As described in the previous report, we found several...
bacteria with trans-4 or cis-3 hydroxylating activity among 3,000 bacteria isolated from soil (13). Further screening of microbes will open the way to detect previously undiscovered hydroxylases of free L-proline.

ACKNOWLEDGMENTS

We thank Ruriko Nishimura and Yasue Watanabe for their technical assistance.

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