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Detection of Novel Proline 3-Hydroxylase Activities in *Streptomyces* and *Bacillus* spp. by Regio- and Stereospecific Hydroxylation of L-Proline

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During the screening of microbial proline hydroxylases, novel proline 3-hydroxylase activities, which hydroxylate free L-proline to free *cis*-3-hydroxy-L-proline, were detected in whole cells of *Streptomyces* sp. strain TH1 and *Bacillus* sp. strains TH2 and TH3 from 3,000 strains isolated from soil. The reaction product was purified from a reaction mixture of *Streptomyces* sp. strain TH1, and its chemical structure was identified as *cis*-3-hydroxy-L-proline by instrumental analyses. Proline 3-hydroxylase activity was also detected in *Streptomyces canus* ATCC 12647 which produces the 3-hydroxyproline-containing peptide antibiotic telomycin. *Bacillus* sp. strains TH2 and TH3 were found to accumulate *cis*-3-hydroxy-L-proline in culture media at 426 and 352 μM, respectively. It was suggested that hydroxylation occurred in a highly regio- and stereospecific manner at position 3 of L-proline because no hydroxylation product other than *cis*-3-hydroxy-L-proline was observed. Proline 3-hydroxylases of these strains were first characterized on crude enzyme preparations. Since 2-oxoglutarate and ferrous ion were required for hydroxylation of L-proline, these 3-hydroxylases were thought to belong to a family of 2-oxoglutarate-related dioxygenases. The reaction was inhibited by Co^{2+}, Zn^{2+}, and Cu^{2+}. L-Ascorbic acid accelerated the reaction. The optimum pH and temperature were 7.5 and 35°C, respectively.

Hydroxyproline is a useful chiral synthon for chemical synthesis of pharmaceuticals. In order to construct an efficient manufacturing process of hydroxyproline, the possibility of enzymatic hydroxylation of L-proline, which is now industrially produced by fermentation, was examined. However, proline hydroxylase activity, which hydroxylates free L-proline to free hydroxyproline, was reported only in *Streptomyces griseoviridis* P8648 (2, 3, 11, 15).

Since the reported activity was so weak that the activity was detected only by using ^{14}C-labeled L-proline as a substrate (15), we have developed (i) a whole-cell reaction system which enables rapid and convenient screening and (ii) a sensitive and hydroxyproline-specific detection method by postcolumn derivatization with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) for the analyses of stereoisomers of hydroxyprolines by high-performance liquid chromatography (HPLC) to obtain microbial proline hydroxylases (16). This method has enabled the detection of hydroxyprolines even at the picomole concentration after separation of all stereoisomers of hydroxyprolines by ligand-exchange chromatography. Microbial proline 4-hydroxylases were then screened by this system from more than 3,000 actinomycete strains isolated from soil, and 5 actinomycete strains, as well as 3 stock strains, that produce proline 4-hydroxylase activities have been found (the results will be published elsewhere). All of these activities apparently required 2-oxoglutarate and ferrous ion for the reaction. While screening proline 4-hydroxylases, we have found another proline hydroxylase activity which hydroxylates free L-proline to free *cis*-3-hydroxy-L-proline.

3-Hydroxyproline has been found in certain proteins such as collagen (1, 10, 13) and in some peptide antibiotics such as telomycin (7, 19, 20) and plusbacin (22). The biosynthetic route to 3-hydroxyproline has been studied for mammalian collagen biosynthesis, in which only peptidyl proline is hydroxylated to peptidyl 3-hydroxyproline by procollagen-proline 3-dioxygenase (EC 1.14.11.7), as in the case of 4-hydroxyproline by procollagen-proline dioxygenase (EC 1.14.11.2) (4, 18). These enzymes belong to a family of 2-oxoglutarate-related dioxygenases, which require 2-oxoglutarate and O_2 as cosubstrates for the reaction (18). On the other hand, the mechanism of formation of 3-hydroxyproline has not been revealed in microbial sources in which 3-hydroxyproline-containing peptide antibiotics have been produced.

Here we report the detection and preliminary characterization of microbial proline 3-hydroxylase activities, which hydroxylate free L-proline to free *cis*-3-hydroxy-L-proline. This is the first report of proline 3-hydroxylase activities.

MATERIALS AND METHODS

Chemicals. *trans*-4-Hydroxy-L-proline was purchased from Nacalai Tesque (Kyoto, Japan). *cis*-3-Hydroxy-DL-proline was chemically synthesized by previously published methods (5, 6). NBD was purchased from Nacalai Tesque.

Culture media. HT medium was used for plate cultures. HT medium consists of 10 g of soluble starch per liter, 2 g of NZ amine type A (Wako Pure Chemical Industries Ltd., Osaka, Japan) per liter, 1 g of Bacto Yeast Extract (Difco Laboratories, Detroit, Mich.) per liter, 1 g of meat extract (Kyokuto Seiyaku Kogyo Co., Tokyo, Japan) per liter, and 15 g of agar per liter, and the pH was adjusted to 7.2. SR3 medium was used for seed cultures. SR3 medium consists of 10 g of glucose per liter, 10 g of soluble starch per liter, 5 g of Bacto Yeast Extract per liter, 5 g of Bacto Tryptone (Difco Laboratories) per liter, 3 g of meat extract per liter, and 0.5 g of magnesium phosphate per liter, and the pH was adjusted to 7.2 with 6 N NaOH. Df3 medium consists of 50 g of soluble starch per liter, 30 g of corn steep liquor per liter, 0.5 g of KH_2PO_4 per liter, 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter, and 5 g of calcium carbonate per liter, and the pH was adjusted to 7.0 with 6 N NaOH. Df3 medium was used for cultures in 5-liter jar fermentors.

Cultural conditions. Cells grown on a HT plate were inoculated on 10 ml of SR3 medium in a test tube (180 mm long; inner diameter, 25 mm) as the first seed culture. After cultivation at 28°C for 2 days with shaking, the whole culture was transferred to 200 ml of SR3 medium in a 2-liter baffled flask and cultivated at 28°C for 2 days with shaking. The whole culture was then inoculated to 2 liters of Df3 medium in a 5-liter jar fermentor and cultivated for 2 days at 28°C with agitation of 700 rpm and aeration of 1 liter/liter/min. The cells were harvested by

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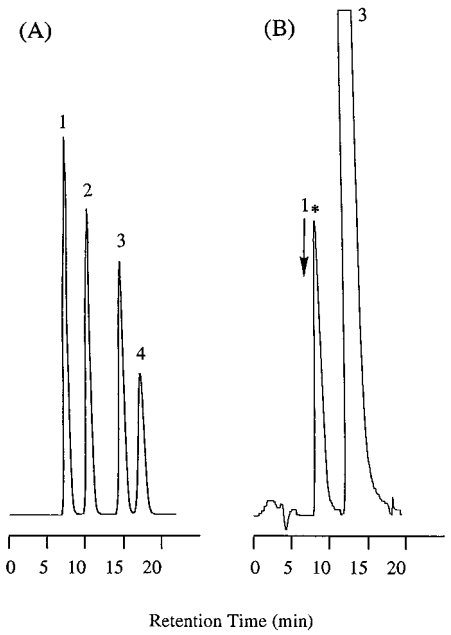

FIG. 1. Detection of *cis*-3-hydroxy-L-proline by HPLC. The HPLC conditions are described in Materials and Methods. (A) A standard mixture containing *trans*-4-hydroxy-L-proline (peak 1), *cis*-4-hydroxy-D-proline (peak 2), L-proline (peak 3), and *cis*-4-hydroxy-L-proline (peak 4) was analyzed by HPLC equipped with a Sumichiral OA-5000 column. (B) The supernatant of the whole-cell reaction mixture of strain TH1 was analyzed by HPLC. The peaks for L-proline (peak 3) and *cis*-3-hydroxy-L-proline (*) were detected by the postcolumn derivatization method with NBD. The arrow indicates the position of *trans*-4-hydroxy-L-proline (peak 1) in a standard mixture.

centrifugation after the culture and washed with cold *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid (TES) buffer, pH 7.5.

Taxonomic analyses. The cultural characteristics of strain TH1 were determined by the methods of the International *Streptomyces* Project (21). Tests for phenotypic characteristics of strains TH2 and TH3 were performed by the methods described by Komagata (9). The morphology of these strains was ascertained by light microscopy and electron microscopy (H7100; Hitachi, Tokyo, Japan). Analyses of isoprenoid quinones and cell wall diamino acids were carried out by the methods described by Tamaoka et al. (24) and Stanek and Roberts (23), respectively.

HPLC analysis. The analyses of hydroxyprolines were carried out by the postcolumn derivatization method with NBD reported previously (16). The samples containing hydroxyproline were analyzed by using the Shimadzu LC-6A HPLC system. This HPLC system was equipped with a Sumichiral OA-5000 column (250 mm long; inner diameter, 4.6 mm; Sumika Chemical Analysis Service Ltd., Osaka, Japan). Chromatographic conditions are as follows. Aqueous copper sulfate solution (1 mM) was used as a mobile phase at a flow rate of 1 ml/min, and the column temperature was kept at 38°C. The injection volume was 10 μl. Borate buffer (0.3 M, pH 9.6) containing 25 mM EDTA was mixed with a mobile phase at flow rate of 0.2 ml/min just after elution through the column. Then NBD solution (1 g/liter in methanol) was mixed at a flow rate of 0.5 ml/min. The reaction mixture was kept at 60°C for a few minutes to derivatize proline and hydroxyproline. NBD derivatives of proline and hydroxyproline formed in the reaction mixture were detected spectrofluorometrically (with an excitation wave length of 503 nm and an emission wave length of 541 nm).

TABLE 1. NMR spectrum data of *cis*-3-hydroxy-L- proline

Position no.[a]	Chemical shift (δ ppm)	
	^{13}C NMR[b]	^{1}H NMR[c] (integration, multiplicity, *J* value [Hz])
2	68.3	4.18 (1H, d, 4.0)
2-COOH	171.3	
3	71.6	4.77 (1H, dt, 1.4, 4.1)
4	33.9	2.27 (1H, dddd, 14.1, 10.8, 9.3, 4.2)[d]
		2.18 (1H, dddd, 14.1, 7.3, 2.8, 1.4)[e]
5	44.5	3.52 (1H, ddd, 11.7, 9.3, 2.8)[d]
		3.62 (1H, dt, 7.3, 11.3)[e]

[a] Position numbers are indicated in Fig. 2.
[b] Recorded at 125 MHz in D_2O.
[c] Recorded at 500 MHz in D_2O.
[d] Position a in Fig. 2.
[e] Position b in Fig. 2.

Whole-cell reaction. The whole-cell reaction was carried out as follows. Cells were collected from culture broth by centrifugation, and then they were suspended in 10 ml of reaction mixture (100 g [wet weight] of cells per liter). The reaction mixture consisted of 5 mM L-proline, 5 mM 2-oxoglutaric acid, 5 mM L-ascorbic acid, 1 mM ferrous sulfate, 100 mM TES buffer (pH 7.5), and 1.4% (vol/vol) of Nymeen solution. Nymeen solution was made up by dissolving 4 g of polyoxyethylene stearylamine (Nymeen S-215; Nippon Oils and Fats Co., Tokyo, Japan) in 10 ml of xylene. The reaction was carried out at 30°C with shaking. Cells were removed by centrifugation after the reaction, and then the amounts of proline and hydroxyproline in the supernatant were determined.

Isolation of *cis*-3-hydroxy-L-proline. Cells (100 g [wet weight]) of *Streptomyces* sp. strain TH1 from 2-day-old culture in a 5-liter jar fermentor were suspended in 1 liter of the reaction mixture, and the reaction was carried out at 30°C for 5 h. After the cells were removed by centrifugation, the pH of the supernatant was adjusted to 4.5 with HCl and applied to a Diaion SK1B column (200 ml, NH_4^+ form; Mitsubishi Kasei Corp., Tokyo, Japan). Fractions containing *cis*-3-hydroxy-L-proline were collected after elution with 0.14 N ammonium hydroxide and concentrated under vacuum. The concentrated solution was then applied to a Diaion PA412 column (20 ml, OH^- form, Mitsubishi Kasei Corp.). Fractions containing *cis*-3-hydroxy-L-proline were collected after elution with 0.01 N HCl and concentrated. Primary amino acids still contaminating the solution were derivatized with *o*-phthalaldehyde to make the separation of *cis*-3-hydroxy-L-proline from primary amino acids possible; i.e., after adjusting the pH of a concentrated solution to 9.6 with NaOH, 10% (vol/vol) *o*-phthalaldehyde (75 mg/ml in ethanol) and 2% (vol/vol) mercaptoethanol (10% aqueous solution) were added to the solution, and the mixture was kept at 60°C for 5 min. The reaction mixture was passed through a SP207 column (10 ml; Mitsubishi Kasei Corp.), and fractions containing *cis*-3-hydroxy-L-proline were collected and concentrated under vacuum. The concentrated solution was applied again to a Diaion PA412 column, and fractions containing *cis*-3-hydroxy-L-proline were collected. After the active fractions were dried, *cis*-3-hydroxy-L-proline was obtained.

Analytical instruments. The ^{13}C nuclear magnetic resonance (NMR) spectrum of the sample in D_2O solution was recorded at 125 MHz and the ^{1}H NMR spectrum of the sample in D_2O was recorded at 500 MHz with a Bruker AM-500 spectrometer (Bruker, Karlsruhe, Germany). Fast atom bombardment-mass spectrometry was done in the positive mode with a JEOL JMS-HX/HX110A mass spectrometer (JEOL, Tokyo, Japan). Optical rotation was measured with a JASCO DIP-370 digital polarimeter (JASCO, Tokyo, Japan).

Preparation of cell extracts. Cells (1 g [wet weight]) were suspended in 5 ml of buffer composed of 50 mM TES (pH 7.5), 10% (vol/vol) glycerol, 2 mM dithiothreitol, and 1 mM EDTA. Cells were disrupted by sonication at 4°C, and the suspension was centrifuged at 4°C at 30,000 × *g* for 30 min. The supernatant was used for the enzyme reactions. Protein in the supernatant was assayed with a Bio-Rad protein assay kit.

Enzyme assay. Proline 3-hydroxylase activity was assayed by measuring the formation of *cis*-3-hydroxy-L-proline from L-proline. The reaction mixture (1 ml) consisted of 5 mM L-proline, 5 mM 2-oxoglutarate, 1 mM ferrous sulfate, 5 mM L-ascorbic acid, 100 mM TES buffer (pH 7.5), and an enzyme preparation. The reaction was carried out at 30°C for 30 min. After the reaction was terminated by heating at 100°C for 2 min, the amount of *cis*-3-hydroxy-L-proline in the mixture was determined. One unit of the activity was defined as the amount of enzyme which catalyzed the formation of 1 nmol of the product in 1 min.

Preparation of acid hydrolysate of the ethylacetate-extracted fraction of the culture broth. Strains were cultured for 3 days at 28°C, and the culture broth was centrifuged at 8,000 × *g* for 30 min at 4°C. The pH of the supernatant was adjusted to 5.0 with HCl for acidic extraction, to 7.0 with HCl or ammonium hydroxide for neutral extraction, and to 9.0 with ammonium hydroxide for alkaline extraction. Then the supernatant was extracted with an equal volume of ethylacetate. The ethylacetate fraction was dried under vacuum and dissolved in 6 N HCl. Acid hydrolysis was done by incubating the HCl solution at 110°C

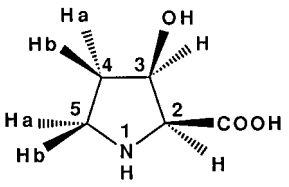

FIG. 2. Structure of *cis*-3-hydroxy-L-proline.

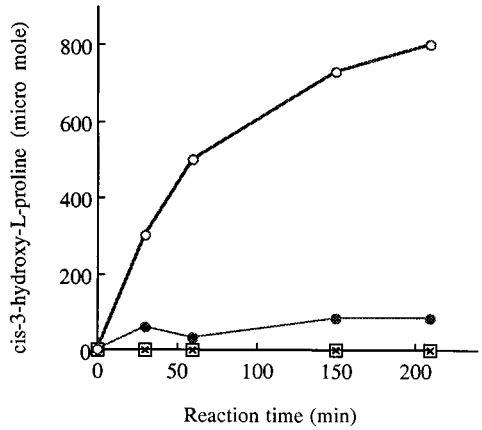

FIG. 3. Time course of the cellular reaction of proline 3-hydroxylase with *Streptomyces* sp. strain TH1. The cellular reactions were done in the mixture containing all the constituents as described in Materials and Methods (○) and in the mixtures lacking ferrous sulfate (●), 2-oxoglutaric acid (×), or Nymeen solution (□) as described in Materials and Methods. The volume of a reaction mixture was 10 ml. Reactions were carried out in test tubes (180 mm long; inner diameter, 25 mm) with shaking at 30°C. The 0.1-ml portions were withdrawn at the times indicated in the figure, and the amount of *cis*-3-hydroxy-L-proline in a reaction mixture was analyzed by postcolumn derivatization method with NBD as described in Materials and Methods.

overnight. The pH of the acid hydrolysate was adjusted to pH 7, and the acid hydrolysate was used for hydroxyproline analyses.

RESULTS

Isolation and identification of *cis*-3-hydroxy-L-proline. During the screening of proline 4-hydroxylases from more than 3,000 microbial strains isolated from soil, the formation of an unusual compound other than *trans*-4-hydroxy-L-proline was observed by the whole-cell reaction on L-proline with three strains, TH1, TH2, and TH3. This compound was detected as a peak on HPLC analysis at 8.3 min after elution through a Sumichiral OA-5000 column by the postcolumn derivatization method with NBD (16) as described in Materials and Methods, while *trans*-4-hydroxy-L-proline was detected at 7.7 min (Fig. 1). Since the derivatization reaction with NBD is specific for imino acid (12, 16), the compound is likely to be a derivative of L-proline.

To identify its chemical structure, the compound corresponding to the peak at 8.3 min was isolated from the reaction mixture with strain TH1 by the ion-exchange chromatographic procedures described in Materials and Methods. From 1 liter of the reaction mixture, 68 mg of the compound was obtained at a yield of 63%. In fast atom bombardment-mass spectrometry analysis of the compound, the molecular ion peak was observed at *m/z* 132 $(M + H)^+$. The optical rotation of the compound was $[\alpha]_D^{21} = -93.4°$ (*c* 0.503, H_2O). NMR data are shown in Table 1. These analytical data showed good agreement with those of *cis*-3-hydroxy-L-proline (Fig. 2) (6, 12, 15, 22). The L configuration of the compound was deduced from the optical rotation. It is reported that L configuration gave minus value and D configuration gave plus value (6), while the compound gave minus optical rotation. The stereochemical relationship between H-2 and H-3 of the compound was deduced from their coupling constant. The coupling constant between H-2 and H-3 was reported to be 4.2 Hz for *cis* and 1.2 or 1.57 Hz for *trans* in the literature (22, 25), while the observed *J* value between H-2 and H-3 was 4.1 Hz (Table 1). The structure was further confirmed by comparison with chemically synthesized *cis*-3-hydroxy-DL-proline (5, 6).

On the basis of these results, it was concluded that *cis*-3-hydroxy-L-proline was formed from L-proline by cellular reaction with strains TH1, TH2, and TH3.

Identification of the strains. Strain TH1 formed aerial mycelium which consisted of moderately short and simple branches arranged in spiral chains of 10 or more ellipsoidal spores. No fragmentation of substrate mycelium was observed, and no sporangia or flagellated spores were formed. The cell wall of the strain contained LL-diaminopimelic acid. On the basis of these taxonomic observations, strain TH1 was identified as a member of the genus *Streptomyces.*

Strains TH2 and TH3 were rod shaped, gram positive, oxidase negative, and catalase positive and formed ellipsoidal endospores. The cell wall amino acid was *meso*-diaminopimelic acid, and the major menaquinone was MK-7 in both strains. The G+C content of the DNA was 36.5 mol% in both strains. On the basis of phenotypic and chemotaxonomic characteristics, strains TH2 and TH3 were identified as members of the genus *Bacillus.*

Cellular activity of *Streptomyces canus* which produces telomycin. *S. canus* ATCC 12647 is known to produce telomycin, which contains both *cis*- and *trans*-3-hydroxy-L-proline as constituents (7, 19, 20). Whole-cell reaction with strain ATCC 12647 was carried out in the same way as that used for strain TH1. Formation of *cis*-3-hydroxy-L-proline was observed by whole-cell reaction; however, *trans*-3-hydroxy-L-proline was not detected in the reaction mixture.

Factors affecting whole-cell reaction. Factors affecting whole-cell reaction were examined on *Streptomyces* sp. strain TH1. Permeabilization of cells by the addition of Nymeen solution to a reaction mixture was essential for whole-cell reaction (Fig. 3). Hydroxylation reaction required 2-oxoglutarate and ferrous ion. Weak activities were observed, however, without the addition of ferrous ion to a reaction mixture, probably because the intracellular pool of ferrous ion was used. Cellular activity was maximum with 4 mM 2-oxoglutate and 1 mM ferrous sulfate. Adding L-ascorbate to a reaction mixture was not necessary for the reaction, but it accelerated the reaction. The optimum pH and temperature of whole-cell reaction were ca. 7.5 and ca. 30°C, respectively. Cellular activity was inactivated completely by heat treatment (100°C, 10 min) of cells.

Cellular reaction with strains TH2, TH3, and ATCC 12647 showed the same requirement for a reaction. These results strongly suggest that the cellular activities of TH1, TH2, and TH3 are caused by 2-oxoglutarate-related dioxygenases. We tentatively referred these activities to proline 3-hydroxylases.

TABLE 2. Proline 3-hydroxylase activities

Strain	Proline 3-Hydroxylase activity		Amt of 3HYP[c] accumulated in the medium (μM)
	Cellular activity[a] (U/g of cells)	Sp act[b] (U/mg of protein)	
Streptomyces sp. strain TH1	101.6	1.19	ND
Bacillus sp. strain TH2	72.7	1.47	426
Bacillus sp. strain TH3	84.1	1.32	352
S. canus ATCC 12647	30.6	0.29	ND

[a] Proline 3-hydroxylase activities measured by whole-cell reactions.
[b] Proline 3-hydroxylase activities measured with cell extracts.
[c] 3HYP, *cis*-3-hydroxy-L-proline. ND, not detected.

TABLE 3. Effects of inhibitors on crude proline 3-hydroxylase activity[a]

Inhibitor added	Concn (mM)	Relative activity (%)
None (standard reaction mixture)		100
EDTA	2.0	5
$CoCl_2$	0.1	18
	1.0	2
$ZnSO_4$	0.1	5
	1.0	0
$CuSO_4$	0.1	41
	1.0	0

[a] The reactions were done with cell extracts of *Streptomyces* sp. strain TH1 by the method described in Materials and Methods.

Cellular proline 3-hydroxylase activities are presented in Table 2.

Activities in cell extracts. Proline 3-hydroxylase activity of strain TH1 was examined in cell extracts. The activity was recovered in a supernatant of the disrupted cell suspension. The activity reached the same maximum as that for the cellular reaction with 4 mM 2-oxoglutarate and 1 mM ferrous sulfate. Adding L-ascorbate to a reaction mixture accelerated the reaction. The optimum pH of the crude enzyme reaction was around 7.5, and the optimum temperature was around 35°C. The hydroxylation reaction by a crude enzyme preparation was inhibited almost completely by the addition of 1 mM (each) Co^{2+}, Zn^{2+}, and Cu^{2+} to a reaction mixture (Table 3). Adding EDTA (2 mM) to a reaction mixture also inhibited the hydroxylation reaction. Crude activities of proline 3-hydroxylases are presented in Table 2.

Stability and ammonium sulfate fractionation of crude enzyme preparation were first examined in *Streptomyces* sp. strain TH1 to obtain information for the enzyme purification. The crude enzyme retained more than 50% of its activity after incubation at 4°C at neutral pH for 1 week. More than 60% of the activity was fractionated by ammonium sulfate between 60 and 70% saturation.

Cofactor requirement of the hydroxylation reaction. 2-Oxoglutarate was strictly required for the hydroxylation of L-proline (Fig. 4). Ferrous ion was also required for the reaction, but the requirement was not as strict in strains TH1, TH2, and TH3, probably because the intracellular pool of ferrous ion was used. L-Ascorbic acid accelerated the reaction of the enzymes from strains TH1, TH2, and TH3.

Detection of *cis*-3-hydroxy-L-proline in culture broth. *Bacillus* sp. strains TH2 and TH3 were found to accumulate *cis*-3-hydroxy-L-proline in the culture medium at concentrations of 426 and 352 μM, respectively (Table 2). No accumulation of free *cis*-3-hydroxy-L-proline in the medium was observed in *Streptomyces* sp. strain TH1 and *S. canus* ATCC 12647. However, 174 μM *cis*-3-hydroxy-L-proline was detected in an acid hydrolysate of ethylacetate-extracted fraction of a 3-day-old culture broth of *Streptomyces* sp. strain TH1. *cis*-3-Hydroxy-L-proline was detected only in the fraction extracted in acidic condition (pH 5.0), not in the fraction extracted in alkaline condition (pH 9.0). No hydroxyproline was also detected in an acid hydrolysate of ethylacetate-extracted fraction of the culture broth of *Bacillus* sp. strain TH2.

DISCUSSION

This is the first report of proline 3-hydroxylase, which hydroxylates free L-proline to *cis*-3-hydroxy-L-proline. Though procollagen-proline 3-dioxygenase (EC 1.14.11.7) has been known to hydroxylate peptidyl L-proline in mammalian systems (4, 18), no proline 3-hydroxylase has been reported before.

The results presented here suggest that proline 3-hydroxylase, which was responsible for the reaction, belongs to a family of 2-oxoglutarate-related dioxygenases, as well as procollagen-proline 3-dioxygenase and procollagen-proline dioxygenase (EC 1.14.11.2), both in mammalian systems and proline 4-hydroxylases in microbial systems (2, 11, 15). While both of the mammalian enzymes accept only peptidyl proline as a substrate, microbial proline hydroxylases catalyze the hydroxylation of free L-proline. However, these enzymes commonly require 2-oxoglutarate and ferrous ion for the hydroxylation reaction. It would be valuable to clarify the structure-function relationships of these enzymes, since Roach et al. (17) have recently reported that a jelly-roll motif is conserved in these 2-oxo-acid-dependent oxygenases.

Hydroxylation was apparently highly regio- and stereospecific. No hydroxylation product other than *cis*-3-hydroxy-L-proline was detected in the reaction with proline 3-hydroxylase or proline 4-hydroxylase. We have detected 4-hydroxylase activities in eight actinomycete strains, but only *trans*-4-hydroxy-L-proline was detected as a hydroxylation product in all cases (data not shown). On the other hand, some stereoisomers of hydroxyprolines exist in nature as constituents of the microbial metabolites such as peptide antibiotics. Therefore, considering the strict specificity of microbial proline 3- and 4-hydroxylase, it is possible that another proline hydroxylase with a different specificity exists.

The physiological role of proline 3-hydroxylase is unclear. The enzyme might be involved in the biosynthesis of peptide antibiotics as in the case of etamycine biosynthesis (8, 14). In fact, *cis*-3-hydroxy-L-proline was detected in an acid hydroly-

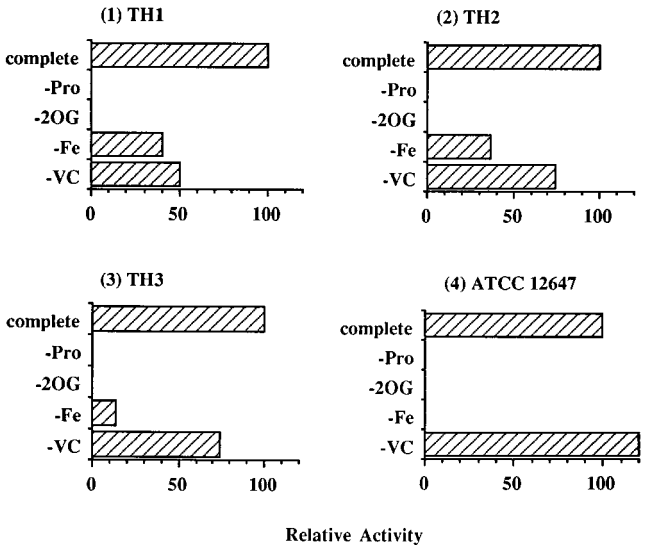

FIG. 4. Requirement for the hydroxylation reaction with proline 3-hydroxylases. Proline 3-hydroxylase activities in various reaction mixtures were measured as indicated in the figure. The standard reaction mixture as described in Materials and Methods (complete) and the reaction mixtures from which L-proline, 2-oxoglutarate, ferrous sulfate, or L-ascorbate was omitted (−Pro, −2OG, −Fe, or −VC, respectively) were used. Relative activities were calculated assuming that the activity in the standard reaction was 100.

sate of the ethylacetate-extracted fraction of the culture broth of strain TH1. The results, as well as the fact that the activity was detected in telomycin-producing *S. canus*, suggest that proline 3-hydroxylase might be involved in the biosynthesis of peptide antibiotics or peptide-like substances which contain hydroxyprolines. However, formation of *trans*-3-hydroxy-L-proline was not observed in *S. canus* ATCC 12647 both by the whole-cell reaction and by the crude-enzyme reaction. Although it is possible to speculate the existence of another hydroxylase or an hydroxyproline epimerase, the biosynthetic mechanism of the formation of *trans*-3-hydroxy-L-proline remains unclear.

Proline 3-hydroxylase would be a useful biocatalyst in the field of organic chemistry and biochemistry as would proline 4-hydroxylase. Although hydroxyprolines are useful chiral synthons for chemical synthesis, only *trans*-4-hydroxy-L-proline is now commercially available and there is no way to obtain hydroxyprolines other than *trans*-4-L isomer. Proline 3-hydroxylase would open the way to the utilization of *cis*-3-hydroxy-L-proline.

We have already purified and characterized the proline 3-hydroxylase from *Streptomyces* sp. strain TH1. The results will be reported elsewhere.

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