Short Communications

Title: Identification and characterization of 2-oxoglutarate-dependent dioxygenases catalyzing selective *cis*-hydroxylation of proline and pipercolinic acid from actinomycetes

Author names: Ryotaro Hara^a, Naoko Uchiumi^b, Kuniki Kino^{a,b,*}

Affiliations: ^a Research Institute for Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan; ^b Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan

* Corresponding author. Tel.: +81 3 5286 3211; fax: +81 3 3232 3889.

E-mail address: kkino@waseda.jp (K. Kino)

Abbreviations: 2-OG, 2-oxoglutarate; Hyp, hydroxproline; HPA, hydroxypipercolinic acid; SrPH, EEP13595; CaPH, ACU72347; SmP4H, L-proline *cis*-4-hydroxylase from *Sinorhizobium meliloti*; FDAA, *N*^a-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide

Abstract

Microbial hydroxylases were screened for the capacity to effect direct hydroxylation of proline and pipecolinic acid, based on genomic information. Of the eight candidates screened, 2-oxoglutarate-dependent hydroxylase from *Streptosporangium roseum* NBRC 3776^T and aspartyl/asparaginyl β -hydroxylase from *Catenulispora acidiphila* NBRC 102108^T showed both proline and pipecolinic acid hydroxylation activities. In the case of L-proline hydroxylation, both enzymes catalyzed the simultaneous formation of *cis*-3-hydroxy-L-proline and *cis*-4-hydroxy-L-proline, and *cis*-4-hydroxy-L-proline was preferentially produced. In the case of L-pipecolinic acid hydroxylation, both enzymes catalyzed the simultaneous formation of *cis*-3-hydroxy-L-pipecolinic acid and *cis*-5-hydroxy-L-pipecolinic acid. While the former enzyme preferentially produced *cis*-3-hydroxy-L-pipecolinic acid, the latter enzyme preferentially produced *cis*-5-hydroxy-L-pipecolinic acid.

Keywords: Hydroxypipecolinic acid, Hydroxyproline, Dioxygenase, Hydroxylase, Hydroxylation, 2-Oxoglutarate

The direct hydroxylation of a compound by using molecular oxygen has attracted much attention in the chemical industry, because it is generally difficult to achieve by a conventional chemical synthesis, as it requires an oxidizing agent or a complex catalyst. In microorganisms, hydroxylation is a common reaction and is usually mediated by a hydroxylase. Generally, hydroxylases play an important role in the primary or secondary metabolism of amino acids, fatty acids, nucleic acids, antibiotics, and aromatic compounds. To date, several classes of hydroxylases, including mono- and dioxygenases, have been known. Among these enzymes, microbial 2-oxoglutarate (2-OG)-dependent dioxygenase is considered an attractive candidate for industrial use, because it does not require heme or a redox partner protein that is essential for other enzymes such as cytochrome P450 monooxygenase, and does not use NAD(P)H, which is rather expensive for commercial use, as a cofactor. Instead, 2-OG-dependent dioxygenase uses non-heme iron(II) as a cofactor and 2-OG as a cosubstrate for the activation of molecular oxygen.

Recent studies on amino acid hydroxylation have shown that diverse microbial hydroxylases, including phenylalanine hydroxylase (Nakata et al., 1979), proline hydroxylase (Hara and Kino, 2009; Lawrence et al., 1996; Mori et al., 1997; Shibasaki et al., 1999a), arginine hydroxylase (Yin and Zabriskie, 2004), asparagine hydroxylase (Strieker et al., 2007), and isoleucine hydroxylase (Kodera et al., 2009), are present in various microorganisms. Interestingly, all these enzymes, except phenylalanine hydroxylase, are 2-OG-dependent dioxygenases. However, the classes of enzymes that catalyze amino acid hydroxylation are relatively restricted, when compared with other classes of hydroxylases or oxygenases that act on other aliphatic and aromatic compounds. Recently, we identified and characterized novel L-proline *cis*-4-hydroxylases from the root nodule bacteria *Mesorhizobium loti* and *Sinorhizobium meliloti*. Based on

our results, a method for the production of hydroxyprolines (Hyps) such as *cis*-3-Hyp, *cis*-4-Hyp, and *trans*-4-Hyp; and hydroxypipicolinic acids (HPAs) such as *cis*-3-HPA, *cis*-5-HPA, and *trans*-5-HPA, in a growing culture of *Escherichia coli* was developed (Huttel and Klein, 2011). In the bioconversion of L-pipicolinic acid by using *E. coli* expressing the L-proline *cis*-4-hydroxylase gene, *cis*-3-HPA and *cis*-5-HPA were produced in the same molar ratio (Huttel and Klein, 2011). *cis*-5-HPA is used as a pharmaceutical intermediate (Bailey and Bryans, 1988) and antifungal agent (Brenner and Romeo, 1986). However, the selective synthesis of *cis*-5-HPA is still difficult owing to the simultaneous production of *cis*-3-HPA. This study was undertaken to screen novel microbial hydroxylases for regioselectivity of L-pipicolinic acid hydroxylation for selective synthesis of *cis*-5-HPA.

While identifying potential microbial hydroxylases for screening, we focused on candidates having some degree of amino acid sequence identity with L-proline *cis*-4-hydroxylase from *S. meliloti* (Smp4H) and the conserved motif that is present within 2-OG-dependent dioxygenases, the 2-His-1-carboxylate facial triad (Koehtop et al., 2005). Based on these criteria, we selected 8 candidate proteins (Table 1). JCM and NBRC strains were obtained from RIKEN Bioresource Center (Ibaraki, Japan), and Biological Resource Center, NITE (Chiba, Japan), respectively. The genomic DNA from the microorganisms was extracted according to a standard protocol (Sambrook and Russell, 2001). Putative hydroxylase genes were amplified by PCR using KOD FX Neo (Toyobo, Osaka, Japan) DNA polymerase. The primer sequences used are listed in Table S1. In a previous study, we cloned, overexpressed, and purified L-proline *cis*-4-hydroxylase from *M. loti* and *S. meliloti* (Hara and Kino, 2009). In brief, *E. coli* Rosetta 2 (DE3) (Merck, Darmstadt, Germany) carrying pET-21d(+) vector (Merck) that contained a hydroxylase gene was cultivated on Luria-Bertani medium (per liter: 10 g

Bacto Tryptone, 5 g Bacto Yeast extract, 10 g NaCl, pH 7.0) supplemented with 50 µg/mL ampicillin and 34 µg/mL chloramphenicol. Recombinant His₆-tagged enzymes were purified with a HisTrap HP column (GE Healthcare, Fairfield, CT) followed by a PD-10 column (GE Healthcare). In the present study, enzyme overexpression and purification was carried out by the same procedure.

Initially, whole-cell reaction was performed to evaluate the hydroxylation activity toward L-proline and L-pipecolinic acid under the following conditions: 5 mM L-proline or L-pipecolinic acid, 10 mM 2-OG, 1 mM L-ascorbic acid, 0.5 mM FeSO₄, and whole cells (OD₆₀₀: 5) in a total volume of 1 mL at 30 °C with vigorous shaking. After 3 h of reaction, the reaction mixture was centrifuged and the supernatant was analyzed by high-performance liquid chromatography. The reaction products were derivatized with N^α-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) and separated by reverse-phase chromatography with UV detection at an absorbance of 340 nm. Hydroxylated products of L-proline and L-pipecolinic acid were detected in the reaction mixtures containing EEP13595 (SrPH) and ACU72347 (CaPH) proteins, indicating that these proteins exhibited L-proline and L-pipecolinic acid hydroxylation activities (Fig. 1). Interestingly, SrPH and CaPH catalyzed the simultaneous formation of *cis*-3-Hyp and *cis*-4-Hyp during L-proline hydroxylation, and *cis*-3-HPA and *cis*-5-HPA during L-pipecolinic acid hydroxylation (Fig. 2).

SrPH and CaPH were then purified and extensively characterized. After enzyme purification, soluble His₆-tagged SrPH and CaPH were obtained, with yields of approximately 6 mg/L and 33 mg/L of *E. coli* culture, respectively. To evaluate their activities on a small scale, appropriate amounts of the purified enzymes were incubated instead of whole cells, under the conditions described earlier. The effects of pH and temperature on enzyme activity were determined by evaluating the formation of

cis-4-Hyp from L-proline at different pH and temperatures. The optimum pH and temperature for SrPH activity were 6.0 and 15°C, and those for CaPH activity were 5.5 and 30°C, respectively.

In the whole-cell reaction, the formation of *cis*-3-Hyp and *cis*-4-Hyp from L-proline, and *cis*-3-HPA and *cis*-5-HPA from L-pipecolinic acid were confirmed, as mentioned above. To assess the ratio of regioisomers in the reaction products in detail, we carried out the reaction with purified enzyme, under the optimum conditions, using Smp4H as a control. When SrPH was used to hydroxylate 5 mM L-proline, 0.75 mM *cis*-3-Hyp and 2.8 mM *cis*-4-Hyp were formed simultaneously, whereas when CaPH was used, 0.44 mM *cis*-3-Hyp and 3.6 mM *cis*-4-Hyp were formed simultaneously. In case of 5 mM L-pipecolinic acid hydroxylation, 2.2 mM *cis*-3-HPA and 0.23 mM *cis*-5-HPA were obtained with the use of SrPH, whereas 0.39 mM *cis*-3-HPA and 1.7 mM *cis*-5-HPA were obtained with the use of CaPH (Fig. 3). In addition, the initial velocities of the enzymes were determined at each optimum condition. In the case of SrPH, the productivities of *cis*-3-Hyp, *cis*-4-Hyp, *cis*-3-HPA, and *cis*-5-HPA were 30.0, 22.7, 13.1, and 8.75 nmol/min/mg protein, respectively, whereas in the case of CaPH, those were 28.1, 38.7, 3.61, and 11.3 nmol/min/mg protein, respectively. These results suggest that both enzymes preferentially hydroxylate the C-4 position rather than the C-3 position of L-proline. During L-pipecolinic acid hydroxylation, SrPH preferentially hydroxylates the C-3 position rather than the C-5 position; however, CaPH preferentially hydroxylates the C-5 position rather than the C-3 position.

In this work, we screened and identified novel L-proline hydroxylases that exhibit novel product specificity from actinomycetes, *S. roseum* and *C. acidiphila*. In case of L-pipecolinic acid hydroxylation, CaPH can be used to obtain a larger proportion of *cis*-5-HPA, whereas SrPH can be used to prepare a larger proportion of *cis*-3-HPA. It

was reported that L-proline *cis*-3-hydroxylase from *Streptomyces* sp. TH1 is also capable of hydroxylating L-pipecolinic acid to produce only *cis*-3-HPA (Shibasaki et al., 1999b). Although this enzyme is ideal for the efficient production of *cis*-3-HPA, there have been no known reports describing the selective *cis*-5 hydroxylation of L-pipecolinic acid. Thus, CaPH has a potential application in the efficient production of *cis*-5-HPA. The mechanisms of substrate recognition and product specificity of hydroxylases remain to be elucidated. In our opinion, the enzymes described here will provide new insights and contribute to the elucidation of these mechanisms.

Acknowledgements

This work was financially supported by Adaptable and Seamless Technology Transfer Program through target-driven R&D, JST (AS231157E). We thank Kyowa Hakko Bio Co. Ltd. for kindly providing us with L-hydroxyprolines and L-hydroxypipecolinic acids and for the helpful discussions.

References

- Bailey, P.D., Bryans, J.S., 1988. Chiral synthesis of 5-hydroxy-(L)-pipecolic acids from (L)-glutamic acid. *Tetrahedron Letters* 29, 2231-2234.
- Brenner, S.A., Romeo, J.T., 1986. Fungitoxic effects of nonprotein imino acids on growth of saprophytic fungi isolated from the leaf surface of *Calliandra haematocephala*. *Applied and Environmental Microbiology* 51, 690-693.
- Hara, R., Kino, K., 2009. Characterization of novel 2-oxoglutarate dependent

162 dioxygenases converting L-proline to *cis*-4-hydroxy-L-proline. Biochemical and
 163 Biophysical Research Communications 379, 882-886.

164 Huttel, W., Klein, C., 2011. A simple procedure for selective hydroxylation of L-proline
 165 and L-pipecolic acid with recombinantly expressed proline hydroxylases.
 166 Advanced Synthesis & Catalysis 353, 1375-1383.

167 Kodera, T., Smirnov, S.V., Samsonova, N.N., Kozlov, Y.I., Koyama, R., Hibi, M., Ogawa,
 168 J., Yokozeki, K., Shimizu, S., 2009. A novel L-isoleucine hydroxylating enzyme,
 169 L-isoleucine dioxygenase from *Bacillus thuringiensis*, produces
 170 (2*S*,3*R*,4*S*)-4-hydroxyisoleucine. Biochemical and Biophysical Research
 171 Communications 390, 506-510.

172 Koehntop, K.D., Emerson, J.P., Que, L., Jr., 2005. The 2-His-1-carboxylate facial triad: a
 173 versatile platform for dioxygen activation by mononuclear non-heme iron(II)
 174 enzymes. Journal of Biological Inorganic Chemistry 10, 87-93.

175 Lawrence, C.C., Sobey, W.J., Field, R.A., Baldwin, J.E., Schofield, C.J., 1996.
 176 Purification and initial characterization of proline 4-hydroxylase from
 177 *Streptomyces griseoviridis* P8648: a 2-oxoacid, ferrous-dependent dioxygenase
 178 involved in etamycin biosynthesis. Biochemical Journal 313, 185-191.

179 Mori, H., Shibasaki, T., Yano, K., Ozaki, A., 1997. Purification and cloning of a proline
 180 3-hydroxylase, a novel enzyme which hydroxylates free L-proline to
 181 *cis*-3-hydroxy-L-proline. Journal of Bacteriology 179, 5677-5683.

182 Nakata, H., Yamauchi, T., Fujisawa, H., 1979. Phenylalanine hydroxylase from
 183 *Chromobacterium violaceum*. Purification and characterization. Journal of
 184 Biological Chemistry 254, 1829-1833.

185 Sambrook, J., Russell, D.W., 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold
 186 Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

187 Shibasaki, T., Mori, H., Chiba, S., Ozaki, A., 1999a. Microbial proline 4-hydroxylase
 188 screening and gene cloning. *Applied and Environmental Microbiology* 65,
 189 4028-4031.

190 Shibasaki, T., Sakurai, W., Hasegawa, A., Uosaki, Y., Mori, H., Yoshida, M., Ozaki, A.,
 191 1999b. Substrate selectivities of proline hydroxylases. *Tetrahedron Letters* 40,
 192 5227-5230.

193 Strieker, M., Kopp, F., Mahlert, C., Essen, L.O., Marahiel, M.A., 2007. Mechanistic and
 194 structural basis of stereospecific cbeta-hydroxylation in calcium-dependent
 195 antibiotic, a daptomycin-type lipopeptide. *ACS Chemical Biology* 2, 187-196.

196 Yin, X., Zabriskie, T.M., 2004. VioC is a non-heme iron, α -ketoglutarate-dependent
 197 oxygenase that catalyzes the formation of 3S-hydroxy-L-arginine during viomycin
 198 biosynthesis. *Chembiochem* 5, 1274-1277.

199

Table 1 Hydroxylase gene candidates screened and characterized in this study.

200

Accession number ^a	Source microorganism	Strain	Annotation	Motif ^b	Identity with SmP4H ^c (%)	Hydroxylase activity
EFV12517	<i>Segniliparus rugosus</i>	JCM 13579 ^T	aspartyl/asparaginyl β -hydroxylase	IIPHRDYLE. .. VHS	33	–
EEP13595	<i>Streptosporangium roseum</i>	NBRC 3776 ^T	2-oxoglutarate-depend ent hydroxylase	IVPHRDFLE ... IHA	30	+
ACU72347	<i>Catenulispora acidiphila</i>	NBRC 102108 ^T	aspartyl/asparaginyl β -hydroxylase	IVPHRDFLE ... IHS	29	+
AEA44416	<i>Fluviicola taffensis</i>	NBRC 106335 ^T	aspartyl/asparaginyl β -hydroxylase	IKPHTDHE L... VHS	26	–
ABG60121	<i>Cytophaga hutchinsonii</i>	NBRC 15051 ^T	conserved hypothetical protein	IKEHTDND T... PHF	23	–
ABQ08010	<i>Flavobacterium johnsoniae</i>	NBRC 14942 ^T	aspartyl/asparaginyl β -hydroxylase	IKEHVDND T... PHS	23	–
EFK57969	<i>Sphingobacterium spiritivorum</i>	NBRC 14948 ^T	aspartyl/asparaginyl β -hydroxylase	IKPHRDPG C... VHW	22	–
ACU63324	<i>Chitinophaga pinensis</i>	NBRC 15968 ^T	aspartyl/asparaginyl β -hydroxylase	IKPHKDPG C... THA	20	–

^a Accession number in the NCBI protein database.^b The bold-typed amino acids indicate essential residues that are conserved in non-heme iron(II) enzymes (ref. 12).^c Calculated by the fast homology search function in GENETYX software ver. 10 using the FASTP program (GENETYX corporation, Tokyo, Japan).

Figure legends

Fig. 1. Analysis of regio- and stereoisomers of hydroxyproline and hydroxypipicolinic acid. (A) L-proline hydroxylation by SrPH, (B) L-proline hydroxylation by CaPH, (C) L-pipicolinic acid hydroxylation by SrPH, (D) L-pipicolinic acid hydroxylation by CaPH. The peak numbers indicate the following compounds: 1, *cis*-4-Hyp; 2, *cis*-3-Hyp; 3, L-proline; 4, *cis*-5-HPA; 5, *cis*-3-HPA; 6, L-pipicolinic acid. Asterisks denote the derivatization reagent, FDAA.

Fig. 2. Proposed reactions catalyzed by SrPH and CaPH. (A) L-proline hydroxylation, (B) L-pipicolinic acid hydroxylation.

Fig. 3. Comparison of product selectivity among SmP4H, SrPH, and CaPH. (A) Amount of *cis*-3-Hyp (unfilled bars) and *cis*-4-Hyp (filled bars), (B) amount of *cis*-3-HPA (unfilled bars) and *cis*-5-HPA (filled bars).

Figure(s)

Fig. 1 Hara et al

Fig. 2 Hara et al

Fig. 3 Hara et al