Synthesis of Mono- and Dihydroxylated Amino Acids with New α-Ketoglutarate-Dependent Dioxygenases: Biocatalytic Oxidation of C–H Bonds


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Iron(II)/α-ketoacid-dependent oxygenases (αKAOs) are enzymes that mainly catalyse hydroxylation reaction. By using a genomic approach combining sequence comparison and protein-domain sharing, a set of 131 αKAO enzymes was prepared. The screening of various substrates revealed five new αKAOs. Four αKAOs were found to be active towards L-lysine, L-ornithine and L-arginine with total regio- and stereoselectivities and yielding the corresponding 3- or 4-hydroxyamino acids. The enzymatic cascade reaction with two stereoselective regiodivergent αKAOs enabled the synthesis of 3,4-dihydroxy-L-lysine.

Introduction

The insertion of an oxygen atom into an unactivated C–H bond is commonly used by nature in biosynthetic pathways; however, it remains a tremendous challenge for chemists. For a long time, cytochrome P450 monoxygenases have attracted attention owing to exceptional oxidative capacities and broad substrate scope as a result of their biological roles. Over the past decade, an increasing number of publications have revealed the diversity of another oxygenase superfamily—the iron(II)/α-ketoacid-dependent oxygenases (αKAOs). Concomitant to the oxidation of the substrate by molecular oxygen, most frequently hydroxylation, these enzymes mediate the oxidative decarboxylation of α-ketoglutarate (αKG) to succinate without the need of any additional cofactor or regenerating enzymatic systems.

αKAOs are involved in various biological processes, for example DNA demethylation, collagen post-translational modifications, or oxidation steps in secondary metabolite biosynthesis. Several bacterial αKAOs have been reported to hydroxylate the side chain of free amino acids or tethered peptides in non-ribosomal peptide biosynthesis. Hydroxylated amino acids contain several stereogenic centres and are thus valuable chiral building blocks for fine chemical synthesis. However, except for the production of hydroxyprolines, which found applications in pharmaceutical and feed industries, with L-proline hydroxylases, the use of αKAOs in industrial biocatalytic processes is infrequent. We herein report the discovery of new dioxygenases active towards free amino acids and related compounds by using a genome-mining approach.

Results and Discussion

Putative αKAOs were selected in a two-step process. Firstly, we selected 274 proteins having sequence identity greater than 30% with a set of experimentally validated αKAOs. Then, we added 56 enzymes depending on the presence of the InterPro motif of proline hydroxylases (IPR007803) or MppO/VioC/AsnO enzymes (IPR014503) catalysing the hydroxylation of amino acids and derivatives in non-ribosomal peptide biosynthesis (Tables S1 and S2). This step led to a collection of 331 candidate αKAOs, including a putative dioxygenase from our model organism Acinetobacter baylyi ADP1. Finally, 131 candidate αKAOs were cloned in an expression vector and heterologously expressed in Escherichia coli strain BL21 with a histidine tag. The panel of tested substrates was organised into 12 pools (Figure 1), which included known substrates, known substrate derivatives, L-amino acids (pools 1–4, 6, and 10–12), together with amine and keto derivatives for which stereoselective hydroxylation could be performed to produce valuable chiral
scaffolds (pools 5 and 7–9). The 131 candidate αKAOs were screened as cell-free extracts against the 12 pools in 96-microwell plates. The dioxygenase activity assay was based on the spectrophotochemical titration of αKG with glutamate dehydrogenase. The proteins demonstrating a significant activity on at least one pool of substrates were purified and studied on individual substrates by using HPLC or GC–MS. Five enzymes demonstrated dioxygenase activity on at least one of the following substrates: hexyl sulphate, lysine, ornithine and arginine. Enzymes catalysing oxidations of arginine and hexyl sulphate with αKAOs have already been described; however, these enzymes are not phylogenetically closely related to those discovered in our screening (Table 1).

**Figure 1.** Pool of substrates.

 Concerning the regioselective hydroxylation of amino acids and derivatives with αKAOs, the C3 regioselectivity is observed much more frequently than the C4 regioselectivity. C4 regioselectivity was observed much more frequently than the C4 regioselectivity.
lective hydroxylation has been reported for aliphatic amino acids and proline, whereas an αKAO dihydroxylation catalysing the conversion of l-arginine into (3R,4R)-3,4-dihydroxy-l-arginine was recently discovered in the streptolidine gene cluster from Streptomyces species; thus, KDO2 and KDO3 are the first C4 regioselective αKAOs that are active towards a polar amino acid such as l-lysine. ODO catalyses the C3 regioselective and (S)-stereoselective hydroxylation of both l-ornithine and l-arginine, as demonstrated by the comparison of NMR data of synthetic products (Scheme 1; also see the Supporting Information for details). Notably, ODO catalyses the hydroxylation of l-arginine with the same regio- and stereoselectivities as of VioC, an αKAO involved in the biosynthesis of viomycin, which is an antibiotic produced by Streptomyces species.

Although new αKAOs are active towards similar substrates, they are not homologous with each other, except for KDO2 and KDO3, which perform the same reaction and share 63% of sequence identity (Table 1). A recent update of our sequence survey revealed the deposit of two homologues of KDO2 and KDO3 in the UniProt Knowledgebase (UniProtKB). Experimental studies revealed their C4 regioselectivity on l-lysine, and the two sequences were, respectively, named KDO4 (G2T8D0) and KDO5 (J3BZS6) (see the Supporting Information for details). However, no sequence similar to that of KDO1 was retrieved.

To investigate their substrate tolerances, KDO1–3 and ODO were screened against d-amino acids and substrate derivatives demonstrating various functional modifications: protection by a methyl ester of α-carboxylic acid, protection by an acetyl group on the α-amino, substitution of the Nε-amino, carboxylic acid in place of the Nε-amino, suppression of the Nε-amino, cyclisation, dimerisation and incorporation of a sulphur atom into the lateral chain. We evaluated the activity on (3S)- and (4R)-hydroxyl-l-lysines (synthesised enzymatically in situ) as well as on (5R)-5-hydroxy-l-lysine; these monohydroxylated l-lysines were precursors of dihydroxy-l-lysines, which were, in turn, highly functionalised chiral scaffolds (Table S3).

For the four αKAOs, the substrate scope was narrow because only closely related amino acid derivatives were found as substrates (Table 2). None of the tested compounds was the substrate of ODO. The three KDOs were active towards 3-, 4- and 5-hydroxy-l-lysines, which gave access to different dihydroxy-l-lysines. Notably, 3,4-dihydroxy-l-lysine was obtained through the cascade reaction with KDO1 and KDO3. The reaction was performed on a preparative scale, which yielded 53% of (3R,4R)-3,4-dihydroxy-l-lysine as γ-lactone (Scheme 2).

<table>
<thead>
<tr>
<th>Table 1. αKAOs from enzymatic screening.</th>
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<tbody>
<tr>
<td>Enzyme ID</td>
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<tr>
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<tr>
<td>KDO1 C7Q42</td>
</tr>
<tr>
<td>KDO2 C7PLM6</td>
</tr>
<tr>
<td>KDO3 A5FF23</td>
</tr>
<tr>
<td>ODO C7Q942</td>
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<td>AtsK Q6FBW1</td>
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[a] With VioC (Q6WZB0) from Streptomyces vinaceus; [b] C7PLM6 and A5FF23 shared 63% of sequence identity; [c] With AtsK (Q9WWU5) from Pseudomonas putida.

| Scheme 1. Biocatalytic synthesis of hydroxylated amino acids. (i) αKAO, substrate (0.1 mmol), αKG (1.5 equiv.), ascorbate (0.25 equiv.; except for ODO: none), Mohr’s salt (0.1 mmol), HEPES (50 mM, pH 7.5), room temperature, 18 h, 300 rpm. (ii) Boc2O (2 equiv.), 5 % NaOH/EtOH (1:1), room temperature, 4 h. (iii) HCl (4 m) in dioxane solution, room temperature, 1 h. 1-2HCl: quantitative yield; 3-2HCl: quantitative yield. |

<table>
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<th>Table 2. Positive results from the substrate promiscuity survey.</th>
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<tr>
<td>Substrate</td>
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<tr>
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<tr>
<td>thialysine 34</td>
</tr>
<tr>
<td>(3S)-3-hydroxy-l-lysine 34</td>
</tr>
<tr>
<td>(4R)-4-hydroxy-l-lysine 34</td>
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<tr>
<td>(5R)-5-hydroxy-l-lysine</td>
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Conclusions

Our genome-mining approach led to the discovery of four new α-ketoacid-dependent oxygenases hydroxylation the side chains of basic amino acids. Two of them, KDO2 and KDO3 (i.e., L-lysine dioxygenases), demonstrated a C3 regioselectivity rather than the C3 regioselectivity frequently encountered for the hydroxylation of amino acids and derivatives. To investigate the C3/C4 regioselectivity of the hydroxylation reaction of amino acids, structural studies combining X-ray crystallography, homology modelling and directed mutagenesis are underway.

Despite a limited substrate range, the new enzymes are of interest for biocatalytic purposes because they proved to be totally regio- and stereoselective. A highly efficient enzymatic synthesis of hydroxy-L-ornithine and mono- and dihydroxy-L-lysine was performed with new α-ketoacid-dependent oxygenases. These chiral scaffolds are of synthetic value in the preparation of more complex functionalised chiral molecules such as natural products and analogues.

Experimental Section

Selection of candidate enzymes

A set of 11 αKAOs related to published experimental data was prepared. This set was used for protein-versus-protein alignments with the BL2 option (BLAST allowing gaps) and a BLOSUM62 scoring matrix against the UniProtKB. Identities restricted to those present by using the Primer3 program. Oligonucleotides were obtained from Sigma-Genosys (St Quentin Fallavier, France). Specific extensions were added to the primers for cloning into the plasmid pET-22b(+) (Novagen, VWR, Fontenay-sous-Bois, France) modified for ligation-independent cloning. The forward primers introduced a hexahistidine sequence in the proteins after the initial methionine for purification purposes. Plasmids were then introduced into E. coli BL21(DE3)pLySE strains (Invitrogen, Life technologies SAS, Saint Aubin, France) for overexpression. KDO4 and KDO5 genes optimised for synthetic gene expression in E. coli were purchased from Genewiz (SigmA Aldrich, Saint Quentin Fallavier, France) and cloned into the modified pET-22(+) with a histidine tag. Cell cultures, isopropyl β-thiogalactopyranoside induction for protein production, and cell extraction were performed as reported previously. For the production of the enzymes used for the initial screening, an adaptation to 96-microwell plate conditions was needed. Briefly, a cell culture (1.6 mL) was induced for recombinant protein production in each well. After centrifugation, cells were suspended in potassium phosphate buffer (300 µL, 50 mM, pH 7.3) containing NaCl (50 mM), 10% glycerol, Pefabloc SC (SigmA Aldrich, Saint Quentin Fallavier, France) (1 mM), and Lysonase bioprocessing reagent (0.2 mL; Merck) and then sonicated in an ultrasonic bath (Branson Ultrasonic Cleaner). After centrifugation to clarify the cell extract, the supernatants were analysed by using sodium dodecyl sulphate polyacrylamide gel electrophoresis to check for recombinant protein production. Protein concentrations were determined by using the Bradford method with bovine serum albumin as the standard (Bio-Rad). The samples were stored at –80 °C.

The enzymes were purified by loading the crude extract onto a NNTA column (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. Large-scale purifications were performed by using nickel affinity purifications, as reported previously. Protein concentrations were determined by using the Bradford method. The samples were analysed by using sodium dodecyl sulphate polyacrylamide gel electrophoresis with the Invitrogen NuPAGE electrophoresis system. The purified proteins were stored at –80 °C.

High-throughput enzymatic assay

All the reactions were performed in 96-microwell plates. The enzymatic reactions were performed with a mixture of αKG (220 µL, 1 mM), sodium ascorbate (2.5 mM), Mohr’s salt (1 mM), cell lysate (from 0.04 to 0.54 mg mL⁻¹ of protein), substrate (5 mM) in N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES) buffer (50 mM, pH 7.5) at RT for 18 h. Each well (90 µL) was transferred to a 96-microwell daughter plate. Then, a mixture (130 µL) containing NADH (600 µM), NH₄Cl (50 mM), glutamate dehydrogenase (18.18 µg mL⁻¹) in tris(hydroxymethyl)aminomethane (TRIS) buffer (50 mM, pH 7.5) was added. The oxidation of NADH was monitored to evaluate the remaining αKG. The enzymatic reaction was performed in 250 mL glass Erlenmeyer flask with a total volume of 4 x 10 mL. Water (4.8 mL), HEPES buffer (pH 7.5, 50 mM), L-lysine (10 mM), αKG (15 mM), sodium ascorbate (2.5 mM), ammonium iron(II) sulphate hexahydrate (1 mM)
were poured into the Erlenmeyer flask. The reaction was started by the addition of an enzyme (final concentration: 0.024 mg/mL). The reaction mixture was orbitally shaken (300 rpm) at RT for 18 h. The conversion (94%) was determined by HPLC analysis after derivatization with fluorenylmethoxycarbonyl chloride (see the Supporting Information for details).

The assembled reaction mixtures were first acidified with trifluoroacetic acid (200 µL) and then centrifuged. The supernatant was collected and freeze dried. The crude product was diluted in water solution (400 µL, 0.8 mmol, 2 equiv.), and the solution was cooled to 0°C. Di-tert-butyl dicarbonate (Boc₂O; 4 mL) was added dropwise to the solution, and the resulting mixture was stirred at RT for 4 h. Water (3 mL) was added, and the aqueous layer was extracted with petroleum ether (3 × 3 mL) to eliminate the excess of Boc₂O. The aqueous layer was acidified with HCl (1 mol/L) until pH 2 and extracted with ethyl acetate (3 × 3 mL). The assembled organic layers were dried over anhydrous MgSO₄, filtered, and evaporated to obtain yellow oil. Purification by using flash chromatography with the gradient mixture of 85–35% hexane + 0.3% HCO₂H/AcOEt + 0.3% HCO₂H in 20 min afforded the diBoc-hydroxy amino acid.

General method for derivatisation with the tert-butoxycarbenyl group on the hydroxy amino acid

The hydroxy amino acid (1 equiv.) was solubilised in HCl (4 mol/L) in dioxane solution (345 µL, 1.38 mmol, 11.5 equiv.), and the reaction mixture was stirred at RT for 1 h. The solvent was removed under reduced pressure, and the residue was triturated successively in toluene (3 × 1 mL), diethyl ether (3 × 1 mL), dichloromethane (3 × 1 mL), and petroleum ether (3 × 3 mL) to eliminate the excess of Boc₂O. The aqueous solution (400 µL) of the crude product was collected and freeze dried. The crude product was diluted in minimum water and eluted through a cation-exchange resin (Dowex 50WX8-200) with a pH gradient from HCl (0.1 M) to 3.5% NH₄OH in water solution. The fractions containing the product were assembled, and the solvent was evaporated under reduced pressure to obtain crude hydroxy amino acid.

General method for derivatisation with the tert-butoxy carbonyl group on the hydroxy amino acid

The diBoc-hydroxy amino acid (1 equiv.) was solubilized in 10% NaOH in water solution (400 µL, 0.8 mmol, 2 equiv.), and the solution was cooled to 0°C. Di-tert-butyl dicarbonate (Boc₂O; 4 mL) in ethanol (400 µL, 1.6 mmol, 4 equiv.) was added dropwise to the solution, and the resulting mixture was stirred at RT for 1 h. The solvent was removed under reduced pressure, and the residue was triturated successively in toluene (3 × 1 mL), diethyl ether (3 × 1 mL), dichloromethane (3 × 1 mL), and petroleum ether (3 × 3 mL) to eliminate the excess of Boc₂O. The aqueous layer was acidified with HCl (1 mol/L) until pH 2 and extracted with ethyl acetate (3 × 3 mL). The assembled organic layers were dried over anhydrous MgSO₄, filtered, and evaporated to obtain yellow oil. Purification by using flash chromatography with the gradient mixture of 85–35% hexane + 0.3% HCO₂H/AcOEt + 0.3% HCO₂H in 20 min afforded the diBoc-hydroxy amino acid.

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Keywords: amino acids - biocatalysis - dioxygenase - hydroxylation - regioselectivity

l-lysine to (3S)-hydroxy-l-lysine, RHEA:40748; l-lysine to (4R)-l-lysine, RHEA:42421; and l-ornithine to (3S)-l-ornithine, RHEA:40932.


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