Biochemical and Biophysical Research Communications 450 (2014) 1458–1461

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Structural optimization of SadA, an Fe(II)- and α-ketoglutarate-dependent dioxygenase targeting biocatalytic synthesis of *N*-succinyl-L-*threo*-3, 4-dimethoxyphenylserine

Hui-Min Qin [a], Takuya Miyakawa [a], Akira Nakamura [a], Makoto Hibi [b], Jun Ogawa [c], Masaru Tanokura [a,*]

[a] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Laboratory of Industrial Microbiology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan
[c] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan

ARTICLE INFO

Article history:
Received 25 June 2014
Available online 10 July 2014

Keywords:
DOPA
DOPS
α-KG turnover activity
Docking simulation
Biocatalyst

ABSTRACT

L-*threo*-3,4-Dihydroxyphenylserine (L-DOPS, Droxidopa) is a psychoactive drug and synthetic amino acid precursor that acts as a prodrug to the neurotransmitters. SadA, a dioxygenase from *Burkholderia ambifaria* AMMD, is an Fe(II)- and α-ketoglutarate (KG)-dependent enzyme that catalyzes *N*-substituted branched-chain or aromatic L-amino acids. SadA is able to produce *N*-succinyl-L-*threo*-3,4-dimethoxyphenylserine (NSDOPS), which is a precursor of L-DOPS, by catalyzing the hydroxylation of *N*-succinyl-3,4-dimethoxyphenylalanine (NSDOPA). However, the catalytic activity of SadA toward NSDOPS is much lower than that toward *N*-succinyl branched-chain L-amino acids. Here, we report an improved biocatalytic synthesis of NSDOPS with SadA. Structure-based protein engineering was applied to improve the α-KG turnover activity for the synthesis of NSDOPS. The G79A, G79A/F261W or G79A/F261R mutant showed a more than 6-fold increase in activity compared to that of the wild-type enzyme. The results provide a new insight into the substrate specificity toward NSDOPA and will be useful for the rational design of SadA mutants as a target of industrial biocatalysts.

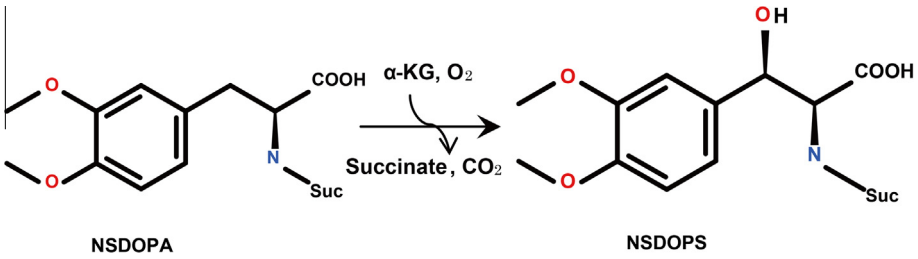
1. Introduction

L-*threo*-3,4-Dihydroxyphenylserine (L-DOPS, Droxidopa) is a psychoactive drug and synthetic amino acid precursor that acts as a prodrug to the neurotransmitters norepinephrine and epinephrine. L-DOPS has been used to treat orthostatic hypotension in patients with pure autonomic failure, multiple system atrophy and Parkinson's disease [1–3]. Since commercial production of L-DOPS by chemical synthesis suffers from complex multistep reactions with protection and deprotection cycles and also increases the environmental burden, the enzymatic approach with high substrate specificity has been accepted as an alternative to the chemical synthesis [4,5]. Although L-DOPS can be directly synthesized using L-threonine aldolase, the products include two different stereoisomers of L-DOPS, and thus the process requires an optical resolution to obtain diastereospecifically pure L-DOPS [6–8]. Currently, it is difficult to enzymatically synthesize L-DOPS on a commercial scale due to the extremely low yields, low stereoselectivity and enzyme instability [5,9].

Dioxygenases are involved in the biological processes ranging from antibiotic biosynthesis to oxygen sensing in humans [10–12]. The enzymes catalyze a number of oxidation reactions. In previous studies, we have reported an Fe(II)- and α-ketoglutarate (KG)-dependent dioxygenase from *Burkholderia ambifaria* AMMD (SadA, 30,664 Da) [13,14]. SadA enantioselectively catalyzes the C3-hydroxylation of not only *N*-succinyl branched-chain L-amino acids but also *N*-succinyl aromatic L-amino acids to produce the hydroxy amino acids. Furthermore, SadA catalyzes hydroxylation of *N*-succinyl-L-3,4-dimethoxyphenylalanine (NSDOPA) to *N*-succinyl-L-*threo*-3,4-dimethoxyphenylserine (NSDOPS), coupled with the conversion of α-ketoglutarate (α-KG) to succinate as illustrated in Scheme 1. Although NSDOPS is useful as a precursor of L-DOPS, the α-KG turnover activity of SadA toward NSDOPA is much lower than that toward *N*-succinyl branched-chain L-amino acids (Fig. 1). Here we applied structure-based modification of SadA to increase its activity toward NSDOPA targeting as an improved industrial catalyst.

* Corresponding author. Fax: +81 3 5841 8023.
E-mail address: amtanok@mail.ecc.u-tokyo.ac.jp (M. Tanokura).

http://dx.doi.org/10.1016/j.bbrc.2014.07.008
0006-291X/



Scheme 1. Enzyme reaction scheme for the SadA-mediated hydroxylation of NSDOPA.

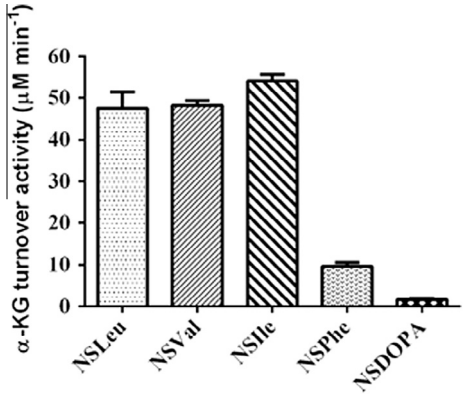


Fig. 1. Enzymatic activity of SadA. The α-KG turnover activity with the five substrates of NSLeu, NSVal, NSIle, NSPhe and NSDOPA were determined. Error bars are standard deviations (n = 3).

2. Materials and methods

2.1. Protein expression and purification

The expression and purification of SadA were performed according to previous reports [14,15]. In brief, *Escherichia coli* Rosetta (DE3) cells (Novagen, Madison, WI) were transformed with the pQE80 plasmid harboring SadA sequence (pQE80-SadA) and were grown in lysogeny broth (LB) medium at 37 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM when the OD_{600} value reached 0.6, and the culture was then further incubated at 25 °C overnight. After harvesting, the cells were disrupted by sonication in the resuspending buffer (20 mM Tris–HCl, pH 8.0, 10 mM imidazole, 0.5 M NaCl and 1 mM dithiothreitol) and the cell debris was removed by centrifugation. SadA was trapped on Ni–NTA Superflow resin (Qiagen, Venlo, Netherlands). After washing, the protein was eluted and the solution containing SadA was concentrated to 10 mg ml^{-1} for the activity assay. Site-directed mutagenesis was performed by PCR with a QuikChange kit (Stratagene, La Jolla, CA) and pQE80-SadA plasmid as a template. The primers used for the mutants are summarized in Supplemental Tables S1–S3. The mutations were confirmed by DNA sequencing. SadA mutants were expressed and purified using the method described above for wild-type SadA.

2.2. Activity assay

Five kinds of substrates—*N*-succinyl-L-leucine (NSLeu), *N*-succinyl-L-valine (NSVal), *N*-succinyl-L-isoleucine (NSIle), *N*-succinyl-L-phenylalanine (NSPhe) and NSDOPA—were synthesized as described previously [13]. The reaction proceeded at 30 °C for 2 h and the reaction mixture was composed of 10 mM substrate, 15 mM α-KG, 0.5 mM $FeSO_4{\cdot}7H_2O$, 10 mM L-ascorbate, 50 mM Tris–HCl buffer (pH 8.0), and 1 mg ml^{-1} purified SadA. The reaction was terminated by the addition of 20 mM EDTA. The activity of SadA was determined based on the production of succinate measured spectrometrically with a succinate test kit (R-Biopharm, Darmstadt, Germany). Enzymatic activity was determined by measuring the amount of succinate produced in the reaction mixture.

2.3. Ligand docking simulations

The initial model of SadA.Zn(II).α-KG.NSDOPA was constructed using the Molecule Builder of the molecular operating environment (MOE; Chemical Computing Group, Montreal, Canada) based on the model of binding between SadA.Zn(II).α-KG and NSPhe [15]. Fe(II) was replaced by Zn(II) in the model based on the crystal structure reported previously [15]. For the binding model of the G79A, G79A/F261W and G79A/F261R mutants, individual residues identified by the MOE site finder search were substituted into the initial model. The final models were obtained by molecular dynamics (MD) simulations that were performed using MMFF94x with the Nose–Poincare–Anderson (NPA) algorithm and the generalized Born method. MD minimization was performed with a time step of 0.001 ps until the model energy was converged.

3. Results and discussion

3.1. Characterization of SadA activity

SadA showed similar levels of α-KG turnover activity, which is an indicator of hydroxylation potential toward several kinds of *N*-substituted branched-chain L-amino acids (NSLeu, NSVal and NSIle), whereas it showed lower α-KG turnover activity toward NSPhe (approximately 30% of the activity toward NSPhe) than toward *N*-succinyl branched-chain L-amino acids (Fig. 1). In addition, SadA showed extremely low activity toward NSDOPA (approximately 17% compared with the activity toward NSPhe).

3.2. Structure-based modification

We previously determined the crystal structure of the SadA.Zn(II).α-KG complex [15]. The active-site residues coordinating the zinc ion were located in a deep cavity of the double-stranded beta-helix (DSBH) fold at the core of the structure and were conserved in the Fe(II)- and α-KG-dependent dioxygenase family [16–18]. Furthermore, Gly79 and Phe261 of SadA was reported to play a pivotal role in the recognition of substrates NSLeu and NSPhe. Based on the NSPhe-binding model [15], we constructed a binding model of NSDOPA in the catalytic cavity of SadA.Zn(II).α-KG (Fig. 2). The cavity is not large enough to accommodate NSDOPA, and the binding of NSDOPA would cause steric hindrance with Gly79 and/or Phe261 (Fig. 2), which is consistent with the low α-KG turnover activity toward NSDOPA (Fig. 1).

In order to relieve the steric interference, Phe261 and Gly79 were selected as the mutation sites. We performed site-directed mutagenesis of Phe261 and screened the mutants that would