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Biochemical and Biophysical Research Communications 295 (2002) 55–61

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C-terminus modification of *Streptomyces clavuligerus* deacetoxycephalosporin C synthase improves catalysis with an expanded substrate specificity

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Received 21 May 2002

Abstract

The biosynthesis of cephalosporins is catalyzed by deacetoxycephalosporin C synthase (DAOCS). Based on computational, biochemical, and structural analyses, it has been proposed that modification of the C-terminus of DAOCS might be a constructive strategy for engineering improvement in enzyme activity. Therefore, five hydrophilic residues namely N301, Y302, N304, R306, and R307 located in proximity to the C-terminus of *Streptomyces clavuligerus* DAOCS (scDAOCS) were selected and each substituted with a hydrophobic leucine residue. Substitutions at positions 304, 306, and 307 created mutant scDAOCSs with improved efficiencies in penicillin analog conversion up to 397%. And since it has been previously advocated that the C-terminus is crucial for guiding substrate entry, a truncated mutant DAOCS was constructed to assess its involvement. The truncation of the C-terminus at position 310 in the wild-type scDAOCS resulted in reduction of indiscriminate conversion of penicillin analog but this defect was compensated by the replacement of asparagine with leucine at position 304.

Keywords: Deacetoxycephalosporin C synthase; C-terminus; Site-directed mutagenesis; Penicillin analogue

The biosynthesis of cephalosporins is mediated by deacetoxycephalosporin C synthase (DAOCS), an iron-dependent and α-ketoglutarate-requiring dioxygenase. This enzyme is responsible for the oxidative ring expansion of the five-membered thiazolidine ring in penicillin to form the six-membered dihydrothiazine ring of cephalosporin [1,2]. Cho et al. [3] showed that DAOCS required ferrous iron, 2-oxoglutarate, molecular oxygen, ATP, and DTT as cofactors to convert penicillin substrates into cephalosporins. Cephalosporins are widely used and are rapidly expanding as a family of antimicrobial chemotherapeutics [4]. Owing to their broad spectrum of activity coupled with low toxicity, cephalosporins have been efficacious in the treatment of many infectious diseases. Furthermore, the advantage of cephalosporins over penicillins is their resistance to penicillin β-lactamases [5]. With these assets, the enzymatic conversion of penicillin G and other penicillin analogs is an important strategy for the manufacturing of new and better oral cephalosporins.

Computational analysis has revealed that N304 is strategically located in the catalytic cavity of *Streptomyces clavuligerus* DAOCS (scDAOCS) and is crucial for enticing different substrates to this center [6]. We have also shown that substitution of this asparagine at position 304 to a hydrophobic leucine residue assists in stabilizing the hydrophobic substrate bound state and consequently improves the biotransformation of penicillin G, as well as two other penicillins, ampicillin and amoxicillin, to their respective cephalosporin moieties. In an earlier report, Lyold et al. [7] revealed that the C-terminus of DAOCS might assist in the introduction of substrates to the active site. Since N304 is located near the C-terminus of scDAOCS, it is conceivable that other hydrophilic residues situated at this terminus might be favorable targets for modification. Therefore, polar residues namely N301, Y302, N304, R306, and R307 located at the C-terminus of scDAOCS, were each mutated to a hydrophobic leucine residue to investigate their influence on the enzyme activity. A311, the last

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0006-291X/02/$ - see front matter
PII: S0006-291X(02)00629-0

amino acid residue of scDAOCS was also truncated to examine its role in flexing the C-terminus. The effects of all the proposed substitution and truncation on the activity of scDAOCS were eventually determined via bioassay and HPLC in this study.

Materials and methods

Materials. Biochemicals of analytical grade were purchased from Amersham Pharmacia Biotech, Sigma–Aldrich, and Bio-Rad Laboratories. HPLC-grade solvents were purchased from Fisher-Scientific. Penicillin G, ampicillin, amoxicillin, phenethicillin, carbenicillin, penicillin V, and metampicillin were purchased from Sigma–Aldrich. *Escherichia coli* strain Ess was provided by Professor Arnold L. Demain from Drew University, New Jersey, USA. The reverse-phase HPLC purified oligonucleotide primers were synthesized by Operon Technologies. Restriction enzymes were purchased from Promega, Statagene, and New England Biolabs. T4 DNA ligase was purchased from BRL. *Pfu* DNA polymerase was purchased from Promega. Penicillinase was purchased from Becton–Dickinson. All enzymes were used according to manufacturer's instructions.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange Site-directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. The recombinant expression vector pscEXP–GST [8] was used as the mutagenesis template. Sequences of the oligonucleotide pairs used for mutagenesis are shown in Table 1. The mutagenic primers were designed to alter the codons of N301, Y302, N304, R306, and R307 to that of leucine. The site-specific nucleotide modifications were analyzed using the ABI PRISM dye terminator cycle sequencing kit and the results verified by ABI 373 automated DNA sequencer as previously described [9].

Construction of recombinant expression vectors. The oligonucleotide primers harboring the *Eco*RI and *Bam*HI restriction enzyme sites were designed to amplify the WT-ΔK310 (wild-type scDAOCS with A311 truncated) and N304L-ΔK310 genes (N304L mutant scDAOCS with A311 truncated) from pscEXP–GST [8] and pscEXP–GST-N304L [6]. The nucleotide sequences of these primers used were OL168: 5′-AGCA GTGAGGATCCATGGACACGA-3′ and OL514: 3′-GCGGCGTGT AGGTTCATCCTTAAGG-5′. PCR amplification with the designed primers was performed to incorporate the selected restriction enzyme sites into the flanking regions of WT-ΔK310 and N304L-ΔK310. The PCR amplified products (~1 kb) were subsequently purified from agarose gels and cloned into pCRII-Blunt-TOPO cloning vector. The TOPO constructs were then digested with appropriate restriction enzymes (*Bam*HI and *Eco*RI) to release the inserted genes. These inserted genes were consequently ligated to the corresponding sites in pGK expression vector [10]. The resultant recombinant pGK constructs carrying the WT-ΔK310 and N304L-ΔK310 genes were then confirmed by digestion with specific restriction enzymes and DNA sequencing (data not shown).

Expression and purification of WT and mutant scDAOCSs. Recombinant clones of WT and mutant scDAOCSs were cultivated to high levels of expression and harvested as previously described [11,12]. Fusion proteins of the WT and mutant scDAOCSs were purified from soluble cell free extracts by affinity chromatography using the MicroSpin GST Purification Module (Amersham Pharmacia Biotech) according to manufacturer's instructions. The protein fractions were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Bio-Rad GS-700 Imaging Densitometer was used to estimate the relative expression levels of WT and mutant scDAOCSs. Protein concentrations were measured by the Bradford assay [13] and bovine serum albumin (Sigma) was used as the reference standard.

Enzyme assays. The standard reaction mixtures reported by Sim and Sim [8] were adopted and designated as Rxn SS in this study. Penicillin G, ampicillin, amoxicillin, phenethicillin, carbenicillin, penicillin V, and metampicillin were added separately to the reaction mixtures to a final concentration of 4 mg/ml. Purified GST–scDAOCS adjusted to a concentration of ~2–3 mg/ml was used for each enzyme assay. Procedures for the addition of reaction components and for the detection of ring expansion activities of WT and mutant scDAOCSs through bioassay were done as previously described [6,8,14]. The agar plate diffusion method [8] was used to detect penicillinase-resistant cephalosporin products and cephalosporin C was used as the reference standard. Aliquots of 200 μl of reaction mixtures were added into each bioassay well. One unit of enzyme activity is defined as the amount of enzyme that forms the equivalent of 1 ng of cephalosporin C per minute under the prescribed conditions.

HPLC analysis. The liquid chromatography system used was a Perkin–Elmer LC system consisting of a PE 785A UV/VIS detector, a PE Series 200 autosampler, a PE Series 200 LC pump and a 5 μm, 250 × 4.6 mm i.d. supelcosil LC-18 column from SUPELCO. Turbo Method Development Software controlled the operation and data acquisition software of the chromatography system. The chromatographic conditions described by Chin et al. [6] was used. Each injection volume was 20 μl and the mobile phase flow-rate was set at 1 ml/min. Separation was effected at ambient temperatures using a mixture of 10 mM KH_2PO_4 at pH 3.0 (adjusted with concentrated phosphoric acid containing 0.06 mM tetrabutylammonium bromide) and methanol (80:20 v/v) in the isocratic mode for 5 min followed by a 15-min linear gradient from 100% of the initial solvent to 100% methanol. Ultraviolet (UV) detection was performed at 260 nm at a sensitivity of 0.02 aufs. Bioassays and HPLC assays were carried out using the same batches of purified proteins for comparison. Since, only the cephem moiety of ampicillin (cephalexin) was commercially available, the conversion of ampicillin was measured via HPLC method. Cephalaxin was used as the standard reference for quantitation.

Computational analysis. DAOCS amino acid sequences were obtained from GenBank databases and aligned using the CLUSTAL W

Table 1
Primers used for site-directed mutagenesis of scDAOCS[a]

Enzyme	Oligonucleotide designation	Sequence of mutagenic primers
N301L	OL515	F 5′ ggatcgggggc**ctc**tacgtgaacatccg 3′
	OL516	R 3′ cctagcccccg**gag**atgcacttgtaggc 5′
Y302L	OL517	F 5′ gatcgggggcaac**ctc**gtgaacatccgc 3′
	OL518	R 3′ ctagcccccgttg**gag**cacttgtaggcg 5′
N304L	OL403	F 5′ ggggcaactacgtg**ctc**atccgccgcac 3′
	OL404	R 3′ ccccgttgatgcac**gag**taggcggcgtg 5′
R306L	OL519	F 5′ ctacgtgaacatc**ctc**cgcacatccaagg 3′
	OL520	R 3′ gatgcacttgtag**gag**gcgtgtaggttcc 5′
R307L	OL521	F 5′ gtgaacatccgc**ctc**acatccaaggca 3′
	OL522	R 3′ cacttgtaggcg**gag**tgtaggttccgt 5′

[a] The mutated codons for the mutagenic primers are shown in bold.

Multiple Sequence Alignment Program (version 1.6) [15]. The protein databank accession number for scDAOCS crystal structures is 1RXG. Modeling, manipulation, and viewing of 3-D structures were performed using the SwissPdb Viewer program version 3.51 (SPdbV) [16,17].

Results

Heterologous expression and purification of WT and mutant scDAOCSs

Mutation in scDAOCS constructs were confirmed by DNA sequencing (data not shown). The WT, N301L, Y302L, N304L, R306L, R307L, WT-ΔK310, and N304L-ΔK310 mutant scDAOCSs were expressed and processed as described previously [6] to yield the respective cell-free extracts for SDS–PAGE analysis (Fig. 1A). High levels of soluble DAOCS proteins (~20% of total soluble protein) were gained as judged from the SDS–PAGE analysis. The proteins obtained were subjected to further purification and bioassay to authenticate the enzyme. It was also noteworthy that none of the amino acid substitutions affected the expression of mutant scDAOCS proteins in *E. coli* BL21 (DE3). The WT and mutant GST–scDAOCSs were purified using Glutathione–Sepharose 4B column chromatography (Fig. 1B) and the purified GST–proteins shown to be of a molecular mass of approximately 60 kDa. These enzymes were found to constitute ~80% of the eluted fraction as estimated by scanning densitometry. The concentrations of purified fusion protein were about 2–3 mg/ml as determined by Bradford assays.

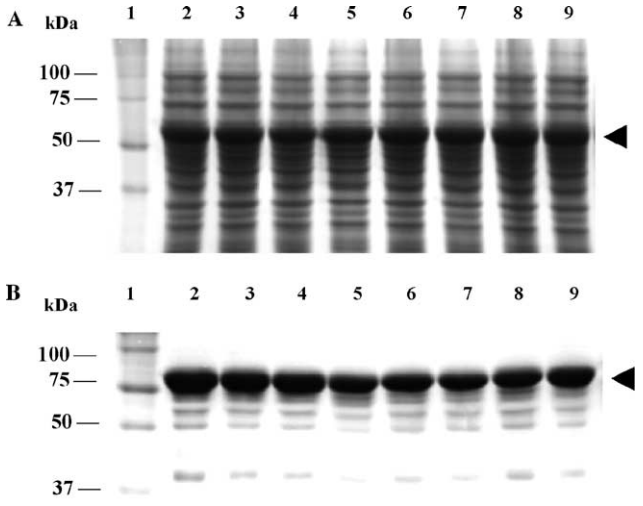


Fig. 1. SDS–PAGE analysis of WT and mutant scDAOCS fusion proteins. (A) Lane 1 shows the prestained molecular mass markers. Lanes 2–9 show the soluble cell free extracts of WT, N301L, Y302L, N304L, N304L, R306L, R307L, WT-ΔK310, and N304L-ΔK310 mutant scDAOCS expressed at 25 °C for 15 h in *E. coli* BL21 (DE3). The arrowhead indicates the position of the GST–scDAOCS fusion proteins. (B) Lane 1 shows the prestained molecular mass markers. The purified WT, N301L, Y302L, N304L, N304L, R306L, R307L, WT-ΔK310, and N304L-ΔK310 mutant scDAOCS fusion proteins are shown in lanes 2, 3, 4, 5, 6, 7, 8, 9, respectively. The arrowhead indicates the position of the GST–scDAOCS fusion proteins.

Determination of hydrophobic penicillin analog conversion via bioassay

The conversion of penicillin analogs by WT and N301L, Y302L, N304L, R306L, R307L, WT-ΔK310, and N304L-ΔK310 mutant scDAOCSs were determined via bioassays using *E. coli* strain Ess as the test organism. All experimental data were confirmed by at least triplicate measurements. The specific activity of WT scDAOCS for the conversion of penicillin G, ampicillin, amoxicillin, phenethicillin, carbenicillin, penicillin V, and metampicillin were determined to be 809, 442, 7, 1472, 83, 31, and 16 U, respectively (Table 2).

Interestingly, the conversion of penicillin analogs for N304L and R306L mutant scDAOCS were significantly enhanced compared to the WT enzyme. As expected, N304L mutant scDAOCS showed an improvement of ~150–400% for the conversion of penicillin substrates compared to WT scDAOCS. R306L also exhibited a drastic improvement of enzyme activity to a level of ~145–330% compared to the WT enzyme (Table 2). The R307L mutant scDAOCS yielded a moderate level of improvement in the conversion of penicillin G, ampicillin, amoxicillin, phenethicillin, and carbenicillin (~110–135%) compared to WT enzyme. However, the conversion of penicillin V and metampicillin for this mutant enzyme was slightly decreased to ~65–90% of the WT activity (Table 2).

The enzyme activity for the N301L and Y302L mutant scDAOCSs also decreased substantially compared to WT enzyme. These mutants retained only ~10% of WT activity for the conversion of most of the substrates tested except for metampicillin. Likewise, the N301L and Y302L mutant scDAOCS retained 69% and 50% WT activity, respectively (Table 2). Interestingly, in the case of the truncated mutants, WT-ΔK310 showed enhanced conversion of penicillin G (124%) compared to WT enzyme but the conversion of other penicillin analogs was decreased to ~70–90% of WT activity. Superfluously, the enzyme activities for N304L-ΔK310 were significantly enhanced to ~155–400% compared to that of WT scDAOCS with augmentation in conversion of all the analogs (Table 2). Nevertheless, the enzyme activity of N304L-ΔK310 mutant scDAOCS is comparable to that of the N304L mutant scDAOCS (Table 2).

Determination of hydrophobic penicillin analog conversion via HPLC

The specific activities of ampicillin conversion for WT, N301L, Y302L, N304L, R306L, R307L, WT-

ΔK310, and N304L-ΔK310 mutant scDAOCS were 2245, 225, 22, 4046, 5253, 2425, 2806, and 4560 U, respectively. N304L, R306L, R307L, WT-ΔK310, and N304L-ΔK310 mutant scDAOCSs showed an improvement in the conversion of ampicillin to a level of 180%, 234%, 108%, 125%, and 203%, respectively, compared to that of WT enzyme (Table 3). The enzyme activities of N301L and Y302L mutant scDAOCS were decreased drastically compared to WT enzyme (Table 3). These results were comparable to those obtained via bioassay whereby the relative activity for penicillin G conversion by WT, N301L, Y302L, N304L, R306L, R307L, WT-ΔK310, and N304L-ΔK310 mutant scDAOCS was detected at levels of 100%, 9%, 7%, 167%, 202%, 124%, 124%, and 154%, respectively (Table 2).

Discussion

N304 is located close to the C-terminus of the scDAOCS. Earlier, we have showed that the mutation of this residue to a hydrophobic leucine enhanced the enzyme activity of this enzyme compared to WT enzyme [6]. While, Lloyd et al. [7] suggested that the C-terminus of DAOCS might assist in the introduction of substrates to the active site, Valegard et al. [1] revealed that shortening of the polypeptide chain of this enzyme from

Table 2
Specific activity of WT and mutant scDAOCSs determined by bioassay

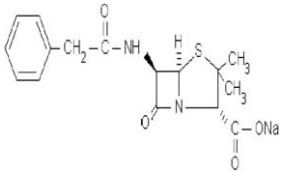
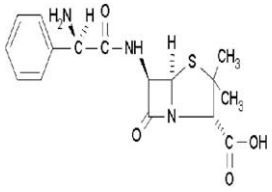
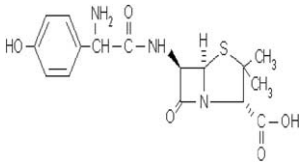
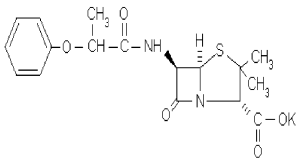
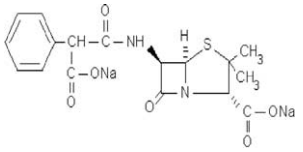
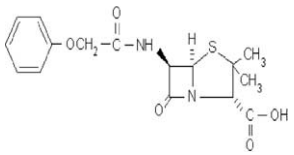
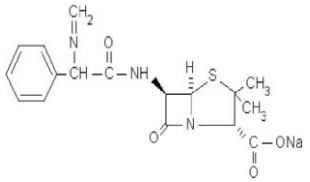
Substrate analog	Enzyme type	Specific activity[a]	Relative activity (%)[b]
Penicillin G 	WT	809 ± 31	100
	N301L	75 ± 20	9
	Y302L	54 ± 7	7
	N304L	1347 ± 188	167
	R306L	1634 ± 362	202
	R307L	1000 ± 162	124
	WT-ΔK310	1001 ± 50	124
	N304L-ΔK310	1249 ± 189	154
Ampicillin 	WT	442 ± 5	100
	N301L	37 ± 22	8
	Y302L	ND	–
	N304L	827 ± 99	187
	R306L	843 ± 239	191
	R307L	590 ± 53	133
	WT-ΔK310	425 ± 60	96
	N304L-ΔK310	727 ± 154	165
Amoxicillin 	WT	7 ± 4	100
	N301L	ND	–
	Y302L	ND	–
	N304L	12 ± 6	171
	R306L	19 ± 7	271
	R307L	8 ± 1	114
	WT-ΔK310	6 ± 2	86
	N304L-ΔK310	12 ± 4	171
Phenethicillin 	WT	1472 ± 31	100
	N301L	168 ± 53	11
	Y302L	30 ± 8	2
	N304L	2692 ± 779	183
	R306L	3404 ± 1113	231
	R307L	1798 ± 497	122
	WT-ΔK310	1025 ± 133	70
	N304L-ΔK310	3169 ± 567	215
Carbenicillin 	WT	83 ± 30	100
	N301L	11 ± 1	13
	Y302L	9 ± 3	11
	N304L	154 ± 48	186
	R306L	221 ± 36	266
	R307L	90 ± 7	108
	WT-ΔK310	55 ± 12	66
	N304L-ΔK310	174 ± 44	210

Table 2 (*continued*)

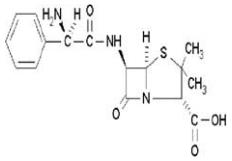
Substrate analog	Enzyme type	Specific activity[a]	Relative activity (%)[b]
Penicillin V 	WT	31 ± 9	100
	N301L	ND	–
	Y302L	ND	–
	N304L	123 ± 2	397
	R306L	102 ± 13	329
	R307L	28 ± 4	90
	WT-ΔK310	22 ± 4	71
	N304L-ΔK310	126 ± 30	406
Metampicillin 	WT	16 ± 4	100
	N301L	11 ± 3	69
	Y302L	8 ± 1	50
	N304L	24 ± 4	150
	R306L	23 ± 5	144
	R307L	10 ± 1	63
	WT-ΔK310	15 ± 4	94
	N304L-ΔK310	33 ± 11	206

ND = not detectable. "–" = values were not quantifiable. Values given are the means ± SD.

[a] One unit of activity is the amount of DAOCS required to form the equivalent of 1 ng cephalosporin C per minute.

[b] Relative specific activity of each mutant enzyme is expressed as the percentage of the specific activity of the mutant enzyme relative to that of the WT enzyme at 100%.

Table 3
Specific activity of WT and mutant scDAOCSs determined by HPLC

Substrate analog	Enzyme type	Specific activity[a]	Relative activity (%)[b]
Ampicillin 	WT	2245 ± 297	100
	N301L	225 ± 35	10
	Y302L	22 ± 3	1
	N304L	4046 ± 157	180
	R306L	5253 ± 432	234
	R307L	2425 ± 300	108
	WT-ΔK310	2806 ± 242	125
	N304L-ΔK310	4560 ± 664	203

[a] One unit of activity is the amount of DAOCS required to form the equivalent of 1 ng cephalexin per minute.

[b] Relative specific activity of each mutant enzyme is expressed as the percentage of the specific activity of the mutant enzyme relative to that of the WT enzyme at 100%.

the C-terminus by six residues diminished the activity of DAOCS. These paradoxical observations warrant inquiry on the importance of the C-terminus arm in assisting the conversion of penicillin substrates.

The results obtained in this study suggest that residues distributed proximately to the C-terminus of scDAOCS affect the enzyme's function. Thus, the substitution of N304 and R306 to leucines drastically enhanced the enzyme activity by ~145–400% compared to WT enzyme while the substitution of R307 exhibited a moderate level of enhancement in substrate conversion (Table 2). In contrast, N301L and Y302L mutant scDAOCSs were drastically reduced in enzyme activity compared to the WT enzyme as shown in Table 2. Similarly, Lee et al. [18] showed that substitution of the tyrosine at position 302 to serine, glutamic acid, and histidine affected 2-oxoglutarate conversions and abolished penicillin G oxidation. These observations were congruent, signifying that residues at position 304 and beyond 307 were subjective to modification, with improvement in analog conversion.

The truncation of A311 in the WT scDAOCS resulted in improved conversion of penicillin G compared to WT scDAOCS but modification of most other penicillin substrates were ruined. After an alteration, the N304L-ΔK310 mutant scDAOCS was as efficacious in penicillin substrate conversion as that of the untruncated N304L mutant enzyme, if not better. For example, the conversion of metampicillin by N304L-ΔK310 mutant enzyme was slightly enhanced compared to N304L mutant enzyme. Lee et al. [19] also showed that the conversion of penicillin N and penicillin G by WT-ΔK310 mutant scDAOCS were enhanced relative to that of the WT enzyme. Although, the truncation of the C-terminus at A311 affected analog conversion, the involved mechanism is unclear. It would seem from this study that the C-terminus of WT scDAOCS can be a hindrance to penicillin G conversion since its truncation enhanced the

activity. The WT-ΔK310 also lost some activities in analog conversion but the replacement of asparagine to leucine at position 304 is able to override the encumbrance.

The distances between the C-6 of ACV to the side chains of N301, Y302, R306, and R307 were approximately 12.58, 11.42, 12.23, and 11.97 Å, respectively, based on the program SPbdV version 3.51. The distance between the C-6 of ACV to the side chains of Y302, R306, and R307 was shortened to 10.89, 8.62, and 8.44 Å after the leucine substitution. Conversely, the distance between the C-6 of ACV to the side chain of N301 was lengthened to 13.86 Å after substitution (data not shown). This observation suggested that the reduction in distance between the side chain of mutated site and ACV to at least 8.5 Å might be preferable for substrate binding and enzyme activity. Since the distance between the C-6 of ACV to the side chain of N304L was approximately equivalent to those of native enzyme at approximately 4.9 Å, it was not surprising to find that the modified mutant performed consistently better than the wild type.

Given that N304L, N306L, and N307L mutant scDAOCS exhibited an enhancement in penicillin conversion, we envisage that the construction of a double mutation or triple mutation in scDAOCS might be additive in creating a mutant scDAOCS with further improvements in enzyme activity. Already, Lee et al. [19] have shown that the truncation or addition of certain amino acids in the C-terminus of scDAOCS created an enzyme with enhanced penicillin N conversion. Therefore, it is also plausible to improve the properties of scDAOCS further by modification of the C-terminus or a combination of mutation and truncation. However, the authentic functionality of the C-terminus in DAOCSs is still unclear. Since the sequence alignment revealed that the fungal cDAOCS/DAC have approximately 20 amino acids more compared to the bacterial DAOCSs [20], the gain in its dual function may have compensated for diversity in analog conversion. Similarly, the DAOCS isolated from *Lysobacter lactamgenus* was found to be longer compared to other bacterial genes [21], but its influence on the enzyme function has also not been investigated.

The conversion of phenethicillin, carbenicillin, and metampicillin by recombinant scDAOCS reported here is novel. Previously, Dubus et al. [22] affirmed that scDAOCS was unable to convert carbenicillin due to the relatively strict selectivity for the side chain of the penicillin substrate. Since carbenicillin can be converted by recombinant WT and mutant scDAOCSs using Rxn SS in this study, the difference in observation could be attributed to this reaction condition used. Lee et al. [19] also proposed that modification on the C-terminus of DAOCS might be a useful strategy for engineering this enzyme to accept unnatural penicillin substrates. In this study, we have demonstrated that it is possible to alter the substrate specificity and enzyme activity of scDAOCS by tailoring the C-terminus of this enzyme. Perhaps, this approach could also be applicable to broaden substrate specificities of other non-heme iron dependent oxygenase and oxidases possessing similar tertiary structures.

Acknowledgment

National University of Singapore research Grant No. R-182-000-019-112 funds this research.

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