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A Complete Library of Amino Acid Alterations at N304 in *Streptomyces clavuligerus* Deacetoxycephalosporin C Synthase Elucidates the Basis for Enhanced Penicillin Analogue Conversion

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N304 of *Streptomyces clavuligerus* deacetoxycephalosporin C synthase was mutagenized to alter its catalytic ability. Given that N304A, N304K, N304L, and N304R mutant enzymes exhibited significant improvements in penicillin analogue conversions, we advocate that replacement of N304 with residues with aliphatic or basic side chains is preferable for engineering of a hypercatalytic enzyme.

Deacetoxycephalosporin C synthase (DAOCS) catalyzes the ring expansion of penicillin to form cephalosporin (3, 4, 8). Numerous modified cephalosporins are widely used, and they are members of a rapidly expanding family of antimicrobial chemotherapeutics (7). The current process for producing 7-aminodeacetoxycephalosporanic acid, a crucial precursor for the production of all oral cephalosporins, involves a multistep, expensive, and polluting chemical ring expansion of penicillin G to phenylacetyl-7-aminodeacetoxycephalosporanic acid. An enzymatic or fermentation process would be cheaper and more environmentally friendly (11); hence, it is essential to engineer *Streptomyces clavuligerus* DAOCS (scDAOCS) with enhanced ring expansion capabilities.

The C terminus of DAOCS has been suggested to be crucial in maintaining the coupling of penicillin oxidation and cosubstrate (α-ketoglutarate) conversion (5, 6). Therefore, modification of this terminus might be useful in instructing DAOCS to accept unnatural penicillin substrates. It has also been suggested that mutational replacements can introduce favorable interactions between ligands and hydrophobic analogues, but eventually, success in forging interactions may be the privilege of selected residues (1, 2). Hence, N304 of scDAOCS was selected for mutagenesis to examine the effect on its catalytic properties. In this study, the use of an expanded library of amino acid residue replacements afforded a detailed verification of the properties of a given side chain.

N304 of scDAOCS was mutated to various amino acids via site-directed mutagenesis. The mutagenized genes were fully sequenced and transformed into *Escherichia coli* BL21(DE3) for protein expression, and the proteins were purified as previously described (1, 2, 3). The standard reaction mixture reported by Sim and Sim (10) was adopted for both hole plate bioassays and high-performance liquid chromatographic analyses in this study. The standard reaction mixture contained 10 mM Tris-HCl (pH 7.4), 0.8 mM ATP, 1.8 mM $FeSO_4$, and 1.28 mM α-ketoglutarate. All penicillin substrates were added separately to the reaction mixtures to a final concentration of 4 mg/ml. Purified enzyme adjusted to a final concentration of 2 mg/ml was used for each assay. The reaction mixtures were incubated at 30°C for 30 min, except for those with metampicillin and amoxicillin, which were incubated for 1 h. An equal volume of methanol was added to stop each reaction. Initially, the catalytic activities of the wild-type (WT) and mutant scDAOCSs for penicillin analogue conversion were measured via hole plate bioassays using Luria-Bertani agar seeded with an overnight culture of *E. coli* Ess and penicillinase to final concentrations of 4.2% (vol/vol) and 50,000 IU/ml, respectively. The cephalosporin product was quantitated by measuring the diameter of zones of growth inhibition on lawns of *E. coli* Ess (10). The specific activities of WT scDAOCS for penicillin G, ampicillin, amoxicillin, phenethicillin, carbenicillin, penicillin V, and metampicillin conversion were determined to be 1,208, 441, 23, 1,491, 89, 27, and 19 U, respectively, where 1 U of enzyme activity is the amount of enzyme that forms the equivalent of 1 ng of cephalosporin C per min under the prescribed conditions.

After replacing N304 of scDAOCS with various aliphatic amino acids, the activities of the expressed enzymes were investigated (Table 1). The N304L mutant scDAOCS showed improvements for all of the penicillin analogue conversions (~130 to 420%). This further supported our earlier observation (1, 2) that changing the asparagine to leucine is instrumental in enhancing interaction with hydrophobic penicillin substrates, thereby contributing to the improvement of its enzymatic activity. Interestingly, N304A mutant scDAOCS also exhibited an improvement in enzyme activity (~160 to 330%). Since changing asparagine to alanine strongly enhanced the ability of the enzyme to convert all of the penicillins used in this study, it suggests that the smaller alanine side chain might have diminished unfavorable steric hindrance, thus providing a somewhat useful spacious accommodation of hydrophobic penicillins in the substrate-binding site. However, the catalytic ability of the N304G, N304I, and N304V mutant scDAOCSs was greatly diminished while that of the N304P mutant scDAOCS was totally abolished. Although proline is very similar to aliphatic amino acids, the rigidity of its ring structure compared with the flexibility of most amino acid side chains

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TABLE 1. Relative activities of WT and mutant DAOCSs determined via bioassay

DAOCS enzyme	Relative activity (%)[a]						
	Penicillin G	Ampicillin	Amoxicillin	Carbenicillin	Phenethicillin	Metampicillin	Penicillin V
WT	100	100	100	100	100	100	100
N304L	129	163	226	235	173	153	415
N304A	163	192	221	242	163	216	333
N304G	42	43	67	37	65	57	60
N304I	29	63	ND[b]	42	58	84	89
N304V	29	43	ND	ND	64	42	89
N304P	ND	ND	ND	ND	ND	ND	ND
N304S	80	62	48	68	80	74	80
N304C	44	24	ND	20	27	43	48
N304T	38	35	35	21	54	54	76
N304M	92	120	139	161	127	137	237
N304F	19	11	ND	ND	23	68	ND
N304Y	22	13	ND	ND	24	39	ND
N304W	20	16	ND	15	15	43	ND
N304D	18	12	ND	15	10	38	ND
N304E	44	24	ND	13	34	48	ND
N304K	242	158	521	404	222	371	733
N304R	263	346	708	280	360	576	493
N304H	60	39	46	28	50	60	36
N304Q	44	76	75	54	67	95	103

[a] The relative 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, which was set at 100%.
[b] ND, not detectable.

might have contributed to difficulties in protein flexing, leading to lost properties (9).

Replacement of N304 with amino acid residues carrying a hydroxyl- or sulfur-containing side chain such as serine, cysteine, and threonine also reduced catalytic ability. However, its replacement with a methionine residue, which also carries a sulfur-containing side chain, resulted in a moderate improvement in enzymatic activities, probably because of its being more hydrophobic. Alteration to aromatic amino acid residues accordingly in N304F, N304Y, and N304W mutant scDAOCSs also resulted in reduced activities compared to that of the WT enzyme. Thus, replacing asparagine with bulky aromatic side chains might have resulted in unfavorable steric hindrances in the catalytic pocket. Likewise, the enzymatic activities of the N304D and N304E mutant scDAOCSs were significantly decreased, suggesting that changing N304 to a negatively charged acidic amino acid was not favorable and might have converted a likely electrostatic attraction into repulsion.

Interestingly, the N304K and N304R mutant scDAOCSs exhibited outstanding enhancement of enzyme activities (up to ~730%), probably because of improved ionic interaction between the positively charged side chain and the prime substrate. A report by Wei et al. also indicated a significant increase in the binding affinity of the enzyme for penicillin G when N304 was replaced with a basic lysine (12). And, although histidine also carries a basic group in its side chain, the N304H mutant scDAOCS showed a marked decrease in enzymatic activity compared to that of the N304K and N304R mutant scDAOCSs. This reflected the trivial basic property of its imidazole side chain compared to lysine and arginine. Furthermore, the side chains of lysine and arginine are positively charged under physiological conditions, thus favoring the charge interaction with the substrate. However, the imidazole side chain of histidine is predominantly in an uncharged state, which probably diminished the substrate conversion rate.

Replacement of asparagine with a structurally similar glutamine (amide) was also done to examine its effect on enzyme activity. Since asparagine and glutamine have similar carbamoyl groups but differ only in amino acid side chain length, the observed results suggested that the longer side chain was not preferred. However, the effect was infinitesimal since the substrate conversion of N304Q mutant scDAOCS was not drastically affected compared to that of the WT enzyme.

For high-performance liquid chromatography analyses, the chromatography condition described by Chin et al. (1, 2) was adopted. Cephalexin, a cephem moiety of ampicillin, was used as a standard reference to quantify the ampicillin conversion of the improved enzymes. Ampicillin and cephalexin were eluted at retention times of approximately 17 and 16 min, respectively. The specific activities of the WT and N304A, N304K, N304L, N304M, and N304R mutant scDAOCSs for ampicillin conversion were 2,916, 5,308, 5,617, 4,574, 3,474, and 5,676 U, respectively; i.e., the N304A, N304K, N304L, N304M, and N304R mutant scDAOCS showed improvements in the conversion of ampicillin to 182, 193, 157, 119, and 195%, respectively, of that of the WT enzyme. These results resembled those obtained via hole plate bioassays.

In conclusion, we showed that replacing N304 with amino acids harboring a strictly aliphatic or basic side chain can incorporate favorable hydrophobic or charge interaction between these mutant enzymes and their prime substrates and thus enhance the efficacy of these enzymes. However, replacement of N304 with residues having bulky side chains was not effective since unfavorable steric hindrance was probably introduced into the catalytic center. Similarly, replacing N304 with acidic amino acids was also ineffective and most likely

hindered by the repulsion between the substrate and the mutagenized ligand.

REFERENCES

1. **Chin, H. S., and T. S. Sim.** 2002. C-terminus modification of *Streptomyces clavuligerus* deacetoxycephalosporin C synthase improves catalysis with an expanded substrate specificity. Biochem. Biophys. Res. Commun. **295:**55–61.
2. **Chin, H. S., J. Sim, and T. S. Sim.** 2001. Mutation of N304 to leucine in *Streptomyces clavuligerus* deacetoxycephalosporin C synthase creates an enzyme with increased penicillin analogue conversion. Biochem. Biophys. Res. Commun. **287:**507–513.
3. **Chin, H. S., J. Sim, K. I. Seah, and T. S. Sim.** 2003. Deacetoxycephalosporin C synthase isozymes exhibit diverse catalytic activity and substrate specificity. FEMS Microbiol. Lett. **218:**251–257.
4. **Cooper, R. D. G.** 1993. The enzyme involved in biosynthesis of penicillin and cephalosporin: their structure and function. Bioorg. Med. Chem. **1:**1–17.
5. **Lee, H. J., M. D. Lloyd, K. Harlos, I. J. Clifton, J. E. Baldwin, and C. J. Schofield.** 2001. Kinetic and crystallographic studies on deacetoxycephalosporin C synthase (DAOCS). J. Mol. Biol. **308:**937–948.
6. **Lloyd, M. D., H. J. Lee, K. Harlos, Z. H. Zhang, J. E. Baldwin, C. J. Schofield, J. M. Charnock, C. D. Garner, T. Hara, A. C. Terwisscha van Scheltinga, K. Valegård, J. A. Viklund, J. Hajdu, I. Andersson, A. Danielsson, and R. Bhikhabhai.** 1999. Studies on the active site of deacetoxycephalosporin C synthase. J. Mol. Biol. **287:**943–960.
7. **Marshall, W. F., and J. E. Blair.** 1999. The cephalosporins. Mayo Clin. Proc. **74:**187–195.
8. **Prescott, A. G., and M. D. Lloyd.** 2000. The iron (II) and 2-oxoacid-dependent dioxygenases and their role in metabolism. Nat. Prod. Rep. **17:**367–383.
9. **Reiersen, H., and A. R. Rees.** 2001. The hunchback and its neighbors: proline as an environmental modulator. Trends Biochem. Sci. **26:**679–684.
10. **Sim, J., and T. S. Sim.** 2001. *In vitro* conversion of penicillin G and ampicillin by recombinant *Streptomyces clavuligerus* NRRL 3585 deacetoxycephalosporin C synthase. Enzyme Microbial Technol. **29:**240–245.
11. **Velasco, J., J. L. Adrio, M. A. Moreno, B. Diez, G. Soler, and J. L. Barredo.** 2000. Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum.* Nat. Biotechnol. **18:**857–861.
12. **Wei, C. L., Y. B. Yang, W. C. Wang, W. C. Liu, J. S. Hsu, and Y. C. Tsa.** 2003. Engineering *Streptomyces clavuligerus* deacetoxycephalosporin C synthase for optimal ring expansion activity toward penicillin G. Appl. Environ. Microbiol. **69:**2306–2312.