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The Effect of Cysteine Mutations on Recombinant Deacetoxycephalosporin C Synthase from *S. clavuligerus*

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Cysteines 100, 155, and 197 of recombinant deacetoxycephalosporin C synthase were mutated to alanine residues. The C100A mutant had properties similar to those of the wild-type enzyme, but mutation of Cys-155 and Cys-197 reduced enzyme activity with penicillin N and penicillin G to different extents.

Key Words: **altered substrate specificity; cephem antibiotic biosynthesis; cysteine mutants; iron(II), 2-oxoglutarate oxygenase; penicillin expansion.**

Penicillin and cephalosporin antibiotics are produced either by direct fermentation or by modification of fermented materials. The key enzymes in the biosynthesis of penicillins and cephalosporins are isopenicillin N synthase (IPNS) and deacetoxycephalosporin C synthase (DAOCS), respectively. Isopenicillin N synthase (IPNS) catalyses the oxidation of L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to give isopenicillin N, which is subsequently epimerised to penicillin N. Deacetoxycephalosporin C synthase (DAOCS) then catalyses the ring expansion of penicillin N to produce DAOC, the first formed cephalosporin (For reviews see (1, 2)). Both IPNS and DAOCS belong to the same sequence-related family of iron(II)-dependent oxygenases/oxidases (3–5), but IPNS is unusual in that it does not use 2-oxoglutarate as a cosubstrate, instead catalysing the four electron oxidation of a single peptide substrate (ACV) (6). Engineering the substrate/product selectivity of DAOCS is of interest from the perspectives of making new antibiotics and improving routes to existing cephem antibiotics [see, for example: (7)]. In the latter regard, modification of DAOCS such that it efficiently ring-expands penicillins with hydrophobic sidechains is a desirable objective.

DAOCS exists as an equilibrium mixture of monomeric and oligomeric forms in solution (8), and aggregation is partially reversed by the addition of DTT (9). Lubbe *et al.* have also reported that native DAOCS from *S. clavuligerus* was inactivated by shaking at 37°C, but that activity could be restored by addition of DTT (10). Problems encountered in obtaining a suitable crystal of DAOCS for structural elucidation led to the speculation that intermolecular disulphide formation may lead to oligomerisation deleterious to the process of obtaining useful crystals. The possibility of improving the cystallisation process using mutant enzymes was thus considered.

DAOCS from *S. clavuligerus* contains seven cysteine residues compared to only two highly conserved cysteines in IPNS (11). Three cysteines, Cys-155, Cys-197 and Cys-226 (numbered with respect to DAOCS from *S. clavuligerus*), are conserved between DAOCS and the closely related oxygenases DACS and DAOC/DACS (3). Cysteine 226 is the only cysteine residue apparently conserved between DAOCS and IPNS (Cys-253 for IPNS from *A. nidulans*), and is similarly located in the IPNS and DAOCS crystal structures, at the end of the fifth β-strand of the jelly-roll motif which constitutes the core of the structures (6, 8).

Neither Cys-106 or Cys-253 in *C. acremonium* IPNS are essential for catalysis as shown by chemical modification and site-specific mutagenesis studies (12). However, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide and DTNB inactivate native DAOCS from *S. clavuligerus*,

Abbreviations used: ACV, L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine; DACS, deacetylcephalosporin C synthase; DAOC, deacetoxycephalosporin C; DAOCS, deacetoxycephalosporin C synthase; DAOC/DACS, deacetoxy/deacetylcephalosporin C synthase; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; G-7-ADCA, phenylacetyl-7-aminodeacetoxycephalosporanic acid; HPLC, high performance liquid chromatography; IPNS, isopenicillin N synthase; IPTG, isopropyl-β-D-thiogalactoside; kDa, kilodaltons; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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suggesting that at least one sulfhydryl group may be important for structural integrity/catalysis (13, 14). Based on the published structure of IPNS-Mn(II) (6), Cys-100, 155 and 197 were identified as residues likely to be on the exterior of DAOCS and candidates to be involved in intermolecular disulphide bond formation. To investigate this hypothesis these cysteine residues were mutated singly and in combination to alanine residues. Whilst the results did not lead to improved crystal forms, they have implications for mutation studies designed to alter the substrate selectivity of DAOCS.

MATERIALS AND METHODS

Materials. All reagents were purchased from the Sigma-Aldrich Chemical Co. or E. Merck, except for: Bradford reagent (Bio-Rad); enzymes for molecular biology [New England Biolabs, except for RNase A (Qiagen) and T7 polymerase (Bohringer Mannheim)]; IPTG (Protech Technology); Hyamine hydroxide (ICN radiochemicals); Optiphase safe (Wallac); Protein chromatography machines/columns and native electrophoreis gels (Amersham Pharmacia Biotech); and HPLC columns (C4, 250 × 4.6 mm) (Phenomenex). All reagents were of at least analytical or molecular biology grade.

Site-directed mutagenesis. The DAOCS gene in the pET-24a expression vector (8) was used for Kunkel mutagenesis (15, 16). Primers designed for the mutants were as follows: C100A, ggtgcccatcgagtaggccatcgagtagtcgga; C155A, ccgcagcagcggctcggcgtcgaggaaggcctc; and C197A, gacgaagccgttggcggcgggtgtctgctgctggat. The entire sequences of all mutants was confirmed by automated dideoxy sequencing (17). All wild-type and mutant plasmids containing the DAOCS gene encode for an isoleucine residue at position 50 as indicated in reference (18). However, other references (19) from the Eli Lilly group, from whom we obtained the gene, show the presence of leucine at this position.

Protein expression, purification and analysis. Recombinant cells were fermented and crude extracts prepared (using 3–5 g cells) as previously reported (8). The filtered sample was loaded onto a Resource Q column (6 mL), which had been equilibrated with 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, and 2 mM DTT, washed with 30 mL of buffer at 10 mL/min, and eluted with a 0–100% 0.3 M NaCl gradient over 40 mL. Fractions (3 mL) were analysed by SDS-PAGE and Bradford analyses. Crystallography grade protein, gel filtration and dynamic laser light scattering analyses and kinetic analyses was produced/carried out as previously reported (8). The mutant proteins were purified in a similar manner to the wild-type enzyme and had a purity of *ca.* 70% (by SDS-PAGE analysis). Native gel electrophoresis was performed using the Phast-system (Amersham Pharmacia Biotech). Kinetic analyses used (at least) duplicate or triplicate measurements which were fitted to the Michaelis-Menten and Lineweaver-Burk equations using the Sigmaplot programme. The identity of the G-7-ADCA produced by the C100A mutant was confirmed by ^{1}H NMR (500 MHz) analysis of the HPLC purified product. Structural models of the various mutants were based on the coordinates for the crystal structure of the DAOCS-2-oxoglutarate complex (8).

2-Oxoglutarate-conversion assays. Assays were modified from the method of Sabourin and Bieber (20). Reaction mixtures in a 5 mL tube contained all cofactors and substrates except for DTT, including [1-^{14}C]-2-oxoglutarate (*ca.* 0.057 μCi/μMol). Hyamine hydroxide (200 μL), in a 0.5 mL Eppendorf tube, was placed above the reaction mixture. Enzyme (*ca.* 1 mg/mL, 100 μL) and DTT (final concentration 4 mM) were used to initiate the reaction, the tube sealed with a rubber septum and incubated at 28°C and 200 rpm. After the required time the reaction was quenched using 20% (v/v) TFA (200 μL) and incubated for 30 min. Conversion was determined by transferring the tube of hyamine hydroxide to a 18 mL scintillation vial containing Optiphase safe (10 mL) followed by scintillation counting (Beckman Instruments). Control incubations utilised buffer in place of enzyme.

TABLE 1

Comparison of DAOCS Activity between Wild-Type Enzyme and Cysteine Mutants with Penicillin N or Penicillin G as Substrates

	Relative activity (%)			
	Penicillin N		Penicillin G	
Enzyme type	HPLC assay	Bioassay	HPLC assay	Bioassay
Wild-type	100	100	100	100
C100A	72	67	100	47
C155A	53	75	<5	0
C197A/A129V	0	0	<5	0
C100A/C155A	64	83	<5	0
C100A/C197A	59	75	<5	0
C100A/C155A/C197A	31	21	<5	0

Note. Activity is normalised to wild-type enzyme. The specific activities of wild-type enzyme with penicillin N was 2.5 nmol/min/mg and with penicillin G 10 nmol/min/mg respectively, detected by HPLC assay and bioassay (8).

RESULTS AND DISCUSSION

Sequencing confirmed that only the desired mutations had been introduced except for the C197A mutant which had an additional mutation, A129V. Circular dichroism analyses suggested the conformations of the mutants were similar to the wild-type protein, except for the C100A/C197A double mutant and the C100A/C155A/C197A triple mutants. A small decrease in absorption at *ca.* 208 nm for these proteins may be indicative of a conformational change.

Activity was measured using both penicillin N and penicillin G as substrates. Kinetic analyses of DAOCS and related enzymes can be complex and the results should be regarded as preliminary (8, 21). Nevertheless, the results are interesting and merit some analysis. The C100A, C155A, C100A/C155A, and C100A/C197A mutants were all significantly active when using penicillin N as a substrate (Table 1). Although reduced levels of activity were observed with all other mutants, these results suggest that Cys-100, Cys-155 and Cys-197 do not play a vital role in catalysis when using penicillin N.

However, the apparent differential effect of the C100A and C155A mutations on penicillin N and penicillin G conversion was unexpected. When penicillin G was used as substrate, only the C100A mutant had a similar activity to the wild-type enzyme. Apparent kinetic parameters for the C100A mutant for penicillin G

were as follows: $K_m = 1.6 \pm 0.3$ mM; $k_{cat} = 0.09 \pm 0.020$ s^{-1}; $k_{cat}/K_m = 56 \pm 15$ M^{-1} s^{-1}. The wild-type enzyme gave the following parameters with penicillin G: $K_m = 1.3 \pm 0.09$ mM; $k_{cat} = 0.10 \pm 0.003$ s^{-1}; $k_{cat}/K_m = 77 \pm 4$ M^{-1} s^{-1}. In contrast, the other cysteine mutants retained less than 5% activity with penicillin G was detected by HPLC analysis, and none by bioassay.

Solution of the DAOCS structure (8, 22) allowed the positions of the mutated cysteines to be identified. Cysteines 100, 155 and 197 in the primary DAOCS sequence correspond to Asp-113, Ser-183 and Val-228 in *A. nidulans* IPNS (6). The crystal structure of DAOCS did not reveal any inter- or intra-molecular disulphide links (22).

Consistent with the levels of activity observed with both penicillins N and G, Cys-100 is located in the first β-strand of the jelly-roll core. This position is close to the surface of DAOCS and a significant distance from the active site (*ca.* 15 Å from the active site iron), as predicted by analysis of the IPNS structure (6).

The C197A/A129V mutant was inactive with penicillin N as a substrate. However, since the C100A/C197A mutant was significantly active, its lack of activity can be attributed to the mutation of A129, which is located on the inner face of the longest α-helix of DAOCS, within the jelly-roll structure. Mutation of Ala-129 to valine may distort the neighbouring β-strand core via unfavourable steric interactions with His-244 and Val-222. His-244 is also adjacent to His-243, which is involved in binding of the iron(II) cofactor at the active site, and thus the A129V mutation probably leads to distortion of the active site.

Cys-197 is close to the surface of DAOCS, a significant distance from active site (*ca.* 16.6 Å from the iron), and is located in the loop joining the third and fourth strand of the jelly-roll core. This is consistent with the significant level of activity when using penicillin N as substrate, but does not explain the lack of activity with penicillin G with the C100A/C197A and C100A/C155A/C197A mutants. To further investigate this phenomenon, the highly purified C100A/C155A/C197A mutant was assayed for 2-oxoglutarate conversion in the absence and presence of penicillin G. In the absence of penicillin G the rate of 'uncoupled' conversion of 2-oxoglutarate for the C100A/C155A/C197A mutant was similar to that observed with wild-type enzyme under the same conditions (2.97 and 6.02 nmol/min/mg, respectively). In the presence of penicillin G the level of 2-oxoglutarate conversion is approximately two-thirds that observed with the wild-type enzyme (19.9 and 31.4 nmol/min/mg, respectively). With recombinant native DAOCS 2-oxoglutarate conversion is tightly coupled with penicillin G conversion in a ratio close to 1:1 (M. D. Lloyd, unpublished results). Since the C100A/C155A/C197A mutant has $<5\%$ of the ring-expansion activity with penicillin G (Table 1), it appears that penicillin oxidation is significantly uncoupled from 2-oxoglutarate conversion. It is unclear whether the C155A, C197A or both mutations is responsible for this effect.

It has been proposed that the uncoupled conversion of 2-oxoglutarate in the presence of substrate may represent an "editing" process to avoid oxidation of incorrect substrates, or oxidation of the correct substrate in an inappropriate way (8). The differential effects of these mutations on the conversion of penicillin N and penicillin G may be a reflection of this editing process, which probably involves conformational changes. However, it can not be entirely ruled out that penicillin G is converted to an alternative product by the C100A/C155A/C197A mutant, which was not detected by the HPLC or bioassay analyses.

Cys-155 is "equivalent" to Ser-183 in IPNS, which it is located reasonably close to the active site (*ca.* 11.6 Å from the iron) facing the ACV substrate. In contrast, Cys-155 is closer to the surface of DAOCS (*ca.* 19 Å from the iron). The DAOCS crystal structures (8, 22) reveals that Cys-155 is proximate to Arg-266 (which is proposed to bind to the carboxylate of penicillin N, (9)) with its sulfhydryl group projecting away from the active site. It is probable that conformational changes occur upon penicillin binding (8), but it seems that interaction of Cys-100 with the penicillin N side-chain is not as important as that of Arg-266 and may be indirect. However, Cys-155 seems to be more important in penicillin G binding. Modification of Cys-155 to a more hydrophobic residue, perhaps in combination with mutation of Arg-266, may be productive in attempts to optimise the binding of penicillin G or other penicillins with hydrophobic side-chains to DAOCS.

Highly purified ($>95\%$ pure by SDS-PAGE analysis) C100A and C100A/C155A/C197A proteins were used in crystallisation experiments. Crystals of the C100A mutant were obtained in a similar manner as for the wild-type enzyme, and had the same *R3* space group and similar unit cell dimensions (8). In contrast the C100A/C155A/C197A mutant failed to crystallise. The latter may reflect a conformational change for this protein as indicated by CD analysis. Analysis of the native DAOCS structure revealed that the Cys-197 is remote from those residues involved in forming the crystallographic trimer. It is not clear which of the three changes in the C100A/C155A/C197A mutant is interfering with the crystallisation process, but the position of Cys-197 suggests that it may reduce the ability of the trimers to pack together in the crystal lattice. Consistent with this theory is the observation that both the C100A and the C100A/C155A/C197A mutants aggregate at a similar level to the wild-type enzyme upon incubation at room temperature, as judged by native gel electrophoresis. As with the wild-type enzyme partial reversal of aggregation was observed on addition of DTT (9). Cys-155 is located relatively close to the active site, which in the crystallographic

trimer binds the C-terminus of the neighbouring molecule. Thus, the C155A mutation may contribute to this phenomenon by interfering with this interaction.

In summary, although modification of Cys-100, Cys-155, or Cys-197 did not lead to an improved crystal form of DAOCS, it led to the discovery of a mutant with an increased selectivity, albeit at a lower level of activity, for penicillin N versus penicillin G. Such a modification is the opposite of a commercially viable protein engineering objective, *i.e.* the selective ring expansion of penicillins with hydrophobic side chains, but suggests that modification of Cys-155 may be profitable in this regard. The results presented herein also suggest that it will be a challenge to maintain full coupling of penicillin G conversion to that of 2-oxoglutarate, even when the mutations are some distance from the active site. It is of interest that active site mutations of the 2-oxoglutarate-dependent oxygenase prolyl 4-hydroxylase have also been shown to increase the proportion of uncoupled conversion of 2-oxoglutarate in the presence of peptide substrates (23).

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