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REVIEW

Directed evolution and rational approaches to improving *Streptomyces clavuligerus* deacetoxycephalosporin C synthase for cephalosporin production

Kian-Sim Goo · Chun-Song Chua · Tiow-Suan Sim

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Abstract It is approximately 60 years since the discovery of cephalosporin C in *Cephalosporium acremonium*. Streptomycetes have since been found to produce the structurally related cephamycin C. Studies on the biosynthetic pathways of these two compounds revealed a common pathway including a step governed by deacetoxycephalosporin C synthase which catalyses the ring-expansion of penicillin N to deacetoxycephalosporin C. Because of the therapeutic importance of cephalosporins, this enzyme has been extensively studied for its ability to produce these antibiotics. Although, on the basis of earlier studies, its substrate specificity was believed to be extremely narrow, relentless efforts in optimizing the in-vitro enzyme assay conditions showed that it is able to convert a wide range of penicillin substrates differing in their side chains. It is a member of 2-oxoglutarate-dependent dioxygenase protein family, which requires the iron(II) ion as a co-factor and 2-oxoglutarate and molecular oxygen as co-substrates. It has highly conserved $HXDX_nH$ and *RXS* motifs to bind the co-factor and co-substrate, respectively. With advances in technology, the genes encoding this enzyme from various sources have been cloned and heterologously expressed for comparative analyses and mutagenesis studies. A high level of recombinant protein expression has also enabled crystallization of this enzyme for structure determination. This review will summarize some of the earlier biochemical characterization and describe the mechanistic action of this enzyme revealed by recent structural studies. This review will also discuss some of the approaches used to identify the amino acid residues involved in binding the penicillin substrate and to modify its substrate preference for possible industrial application.

Keywords Mutational approach · Industrial application · Cephalosporin synthesis · Penicillin · Enzyme improvement

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Introduction

Both penicillin and cephalosporin are important classes of antibiotics. However, the structural relationship between penicillin and cephalosporin was only realized in the late 1950s, about a decade after the discovery of cephalosporin C in *Cephalosporium acremonium* (presently known as *Acremonium chrysogenum*) [1, 39]. Penicillin and cephalosporin possess a four-membered β-lactam ring that binds covalently with nucleophilic active site serine residues in bacterial cell wall biosynthetic enzymes, leading to their inactivation and, indirectly, lysis of the bacterial cell [31, 35, 93]. The basic difference between penicillin and cephalosporin nuclei lies in the additional cyclic structure fused to the β-lactam ring, i.e., penicillin has a five-membered thiazolidine ring and cephalosporin has a six-membered dihydrothiazine ring. Because of this difference, which allows the latter to survive destruction by penicillinase produced by penicillin-resistant bacterial strains, the cephalosporins are generally considered to have a broader antimicrobial spectrum than the penicillins [61]. In addition, a cephalosporin nucleus which has two sites for modification, compared with the penicillin nucleus which only has one, enables the synthesis of a wider variety of cephalosporins.

The production of cephalosporin derivatives stemmed from the ability to synthesize the penicillin nucleus, 6-aminopenicillanic acid (6-APA) and to add different side chains to this nucleus to create a variety of penicillin derivatives with medicinal value [39, 40]. Hence, it was clear that progressive development of various cephalosporin derivatives as useful therapeutics depended on the ability to obtain the cephalosporin nucleus. This was probably one of the factors that initiated interest in deciphering the biosynthetic pathway of cephalosporin. The availability of the cephalosporin nuclei, 7-aminodeacetoxycephalosporanic acid (7-ADCA) and 7-aminocephalosporanic acid (7-ACA), led to the production of many semi-synthetic cephalosporins which differ widely in antimicrobial spectrum and pharmacokinetics [9, 26, 30, 61]. However, these semi-synthetic cephalosporins are expensive, because of high production cost. For example, 7-ADCA is synthesized by multi-step chemical ring-expansion of penicillin G, which is expensive and polluting [86]. This has led to consideration of synthesis of the cephalosporin nucleus by enzymatic means, which is cleaner and more economical [11, 88].

The pathway of penicillin and cephalosporin biosynthesis was initially studied using traditional protein extraction and biochemical characterization methods in the 1970s. With the introduction of recombinant DNA technology, the entire pathway was almost mapped out in the 1990s with many enzymes being characterized; this has been reviewed by several authors [7, 10, 25, 26, 56, 62, 63, 65].

The conversion of penicillin N to deacetoxycephalosporin C, in cell-free extracts of *C. acremonium*, was first reported by Kohsaka and Demain [48]. The enzyme responsible for this reaction, deacetoxycephalosporin C synthase (DAOCS), was purified to near homogeneity from various sources, including *C. acremonium* [27, 50, 77], *Streptomyces clavuligerus* [44, 74], and *Nocardia lactamdurans* [23]. The genes encoding this protein, designated *cef*E from *S. clavuligerus* [49] and *N. lactamdurans* [22], and *cef*EF from *C. acremonium* [76], were cloned and expressed heterologously to aid the biochemical and molecular characterization of DAOCS. The *cef*E gene of the Gram-negative bacterium *Lysobacter lactamgenus* YK90, which produces cephabacin, was also cloned and characterized [46, 47, 66]. This review will summarize some of the earlier characterization of DAOCS, and discuss recent structural studies and protein engineering efforts of this enzyme in the last decade.

DAOCS orthologues from various sources

A few DAOCS genes have been cloned and characterized. Among these, only one is of eukaryotic origin and the rest are from prokaryotes (Table 1). Earlier studies on DAOCS were mainly performed using those from *C. acremonium*, *S. clavuligerus*, or *Nocardia lactamdurans*. Although *S. cattleya* NRRL 8057 [45, 92] and *S. katsurahamanus* T-272 [87] were reported to produce cephamycin C, and *Streptomyces* sp. strain DRS-1 [75] possessed ring-expansion activity, the genes encoding DAOCS from these streptomycetes have yet to be cloned and characterized. Recently, three *cef*E genes from *S. jumonjinensis* [82], *S. ambofaciens*, and *S. chartreusis* [42] were cloned and characterized (Table 1). Although DAOCSs of these three organisms have high protein sequence identities with that of *S. clavuligerus*, they were less active than that of *S. clavuligerus* in converting penicillin G under the respective prescribed assay conditions. Multiple sequence alignment revealed that the amino acid residues in *S. clavuligerus* DAOCS (scDAOCS) reported to be involved in binding penicillin G [42], are highly conserved among various DAOCSs (Fig. 1). This suggests that the binding of penicillin G does not solely depend on these conserved amino acid residues. Furthermore, penicillin G used in these studies is not a natural substrate of these enzymes and their activities in converting their natural substrate have yet to be demonstrated. It is,

Table 1 DAOCS orthologues cloned and characterized

Organism	Genbank accession number	Protein length	Sequence identity[a](%)	Activity	References
S. clavuligerus NRRL 3585	P18548	311	100	Active	[49]
S. jumonjinensis NRRL 5741	AF317908	311	81.7	Active	[15, 82]
S. chartreusis (102SH3)	AY318743	311	76.5	Active	[42]
S. ambofaciens (29SA4)	AY318742	311	81.4	ND[b]	[42]
N. lactamdurans	Z13974	314	70.1	Active	[22]
L. lactamgenus YK90	CAA39984	319	56.1	Active	[46]
A. chrysogenum[c]	P11935	332	53.8	Active	[76]

[a] Primary protein sequence identities were obtained using MatGAT [12] and expressed as percentage of *S. clavuligerus* DAOCS sequence

[b] Not determined

[c] A bifunctional enzyme encoded by *cef*EF, which has both deacetoxycephalosporin C synthase and deacetylcephalosporin C synthase activity

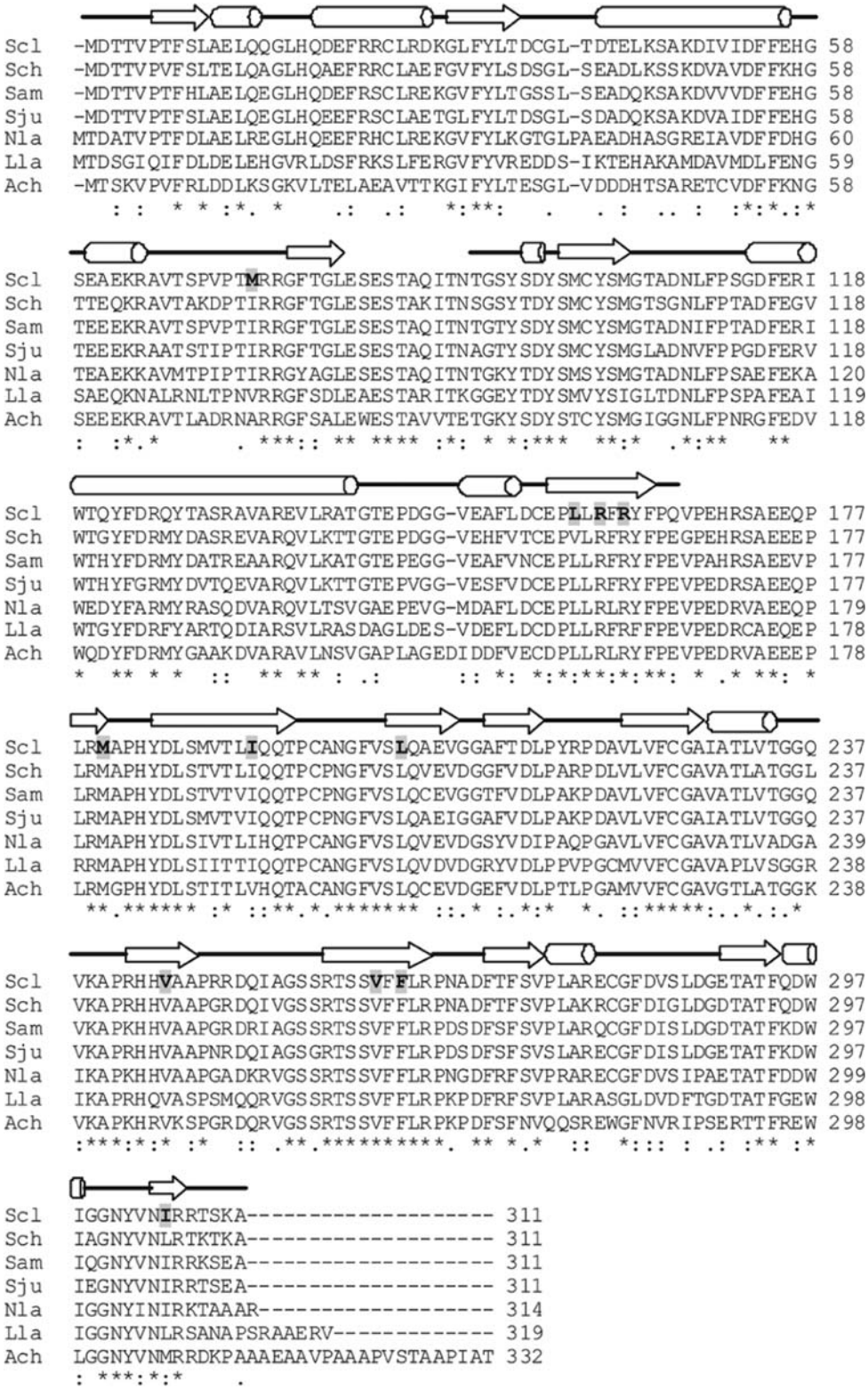


Fig. 1 Multiple sequence alignment of annotated DAOCSs from *S. clavuligerus* NRRL3585 (*Scl*), *S. chartreusis* 102SH3 (*Sch*), *S. ambofaciens* 29SA4 (*Sam*), *S. jumonjinensis* NRRL5741 (*Sju*), *N. lactamdurans* (*Nla*), *L. lactamgenus* YK90 (*Lla*), and *A. chrysogenum* (*Ach*). The accession numbers of these sequences are in Table 2. The substrate binding residues reported by Hsu et al. [42] are highlighted in *gray*. The secondary structure of scDAOCS is presented above its primary sequence on the basis of the structural results reported by Valegård et al. (PDB: 1UNB) [84]. The secondary structures of residues 81–90 and 167–177 were highly disordered and were not determined [84]. The *cylinders*, *arrows*, and *black lines* represent α-helices, β-sheets, and coil structures, respectively

thus, difficult to rationalize the differences in activities observed among the DAOCSs from various sources, especially when scDAOCS is the only enzyme with its three-dimensional structure resolved.

Expression of recombinant *S. clavuligerus* DAOCS

The gene encoding deacetoxycephalosporin C synthase has been cloned into various plasmid vectors for heterologous

expression in *E. coli* [28, 34, 49], *S. lividans* [22, 33], *Penicillium chrysogenum* [13, 24, 68, 70–73], *Pseudomonas putida* [47], and *Pichia pastoris* [3]. High level expression of recombinant scDAOCS has often been carried out in *E. coli* cells together with the use of traditional extraction to purify scDAOCS from *E. coli* cell lysate for biochemical studies [57, 58, 90]. However, this often requires multi-step purification procedures that are time-consuming. Adrio et al. [3] exploited the ability of *P. pastoris* to secrete recombinant proteins to produce high levels of relatively pure recombinant scDAOCS for immobilization studies. This avoids the need to purify the desired protein from the contaminating host proteins. However, ring-expansion activity of the secreted scDAOCS was only detected after 6–12 h of induction of secretion. Sim and Sim used the commonly used glutathione *S*-transferase (GST) to express scDAOCS as a fusion protein [80]. This method enabled an efficient purification of scDAOCS from *E. coli* BL21 (DE3) cells using affinity chromatography.

Biochemical characterization of DAOCS: enzymatic activity and substrate specificity

Enzyme reaction conditions for detection of ring expansion activity have been modified over the last two decades. To achieve optimum conditions for the conversion of penicillin to a cephalosporin, Shen et al. [78] derived a reaction mixture containing 50 mM Tris–HCl (pH 7.4), 10 mM KCl, 10 mM $MgSO_4$, 0.6 mM ascorbic acid, 0.8 mM ATP, 0.04 mM $FeSO_4$, 0.6 mM 2-oxoglutarate, and 0.28 mM penicillin N for detection of the ring-expansion activity of DAOCS from *C. acremonium*. They showed that an effective order of co-factor addition to the reaction mixture is important, i.e., simultaneous addition of iron(II) ion, ascorbic acid, and ATP followed by 2-oxoglutarate and then penicillin N, were obligatory to achieve the highest detectable activity [78]. However, it was found that DAOCS was unable to convert penicillin G and other penicillin N analogs under the optimum conditions described for penicillin N [28]. Maeda et al. [60] tested various conditions such as temperature, duration of reaction, buffers and concentration of penicillin G but were still unable to catalyse the conversion of penicillin G to its respective cephem moiety. They were only able to detect ring expansion activity when penicillin N or D-carboxymethylcysteinyl-6-APA was used as the substrate. As such, DAOCS isolated from various sources was thought to have very narrow substrate specificity [23, 28, 60]. Subsequently, Cho et al. [18] discovered reaction conditions that enabled the ring expansion of penicillin N analogs. This was made feasible by increasing the concentration of iron(II) and the co-substrate, 2-oxoglutarate, in the reaction mixture, which enabled the conversion of penicillin G and 14 other penicillin N analogs to their respective cephem moieties by *S. clavuligerus* NP1 resting cells (Table 2). Thereafter, Sim and Sim showed that omission of KCl, $MgSO_4$, dithiothreitol and ascorbic acid from Cho et al.'s reaction conditions could significantly improve the conversion of penicillin G and ampicillin by GST-scDAOCS fusion protein [80]. Using these improved reaction conditions, Chin and Sim showed that the substrate specificity of scDAOCS is wider than previously thought (Table 2) [17].

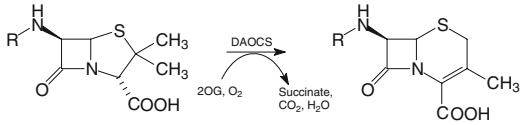
Several assays have been used for detection of DAOCS activity. These include the hole-plate bioassay [16, 80], thin-layer chromatography [43], and high-pressure liquid chromatography (HPLC) [16, 32, 43]. These assays are laborious, cumbersome, and yield limited data points. In addition, these assays are discontinuous and, hence, a large number of samples are required for kinetic studies. A continuous direct spectrophotometric assay for determination of penicillin N conversion by DAOCS was first reported by Baldwin and Crabbe [8]. However, this assay contained sodium azide as one of the components in the reaction mixture, which probably limited its application. In 2001, Dubus et al. [29] reported a revised spectrophotometric assay to probe the kinetic properties and substrate and co-substrate selectivities of the recombinant scDAOCS. They showed that scDAOCS has a K_m value of 0.89 mM and a k_{cat} value of 0.079 s^{-1} for penicillin G conversion, and a K_m value of 4.86 mM and a k_{cat} value of 0.120 s^{-1} for ampicillin conversion. Interestingly, they also showed that the substrate specificity of scDAOCS included acetyl-6-APA, with a K_m value of 3.28 mM and a k_{cat} value of 0.060 s^{-1} for its conversion.

Although many studies have shown that the substrate specificity of scDAOCS is broad, kinetic studies have illustrated its low catalytic efficiency towards penicillin G compared with its natural substrate, penicillin N [53–55, 57, 89, 90]. In a recent study by Wei et al. [89], the K_m and k_{cat} values for penicillin G conversion were reported to be 2.58 mM and 0.0453 s^{-1}, and for penicillin N conversion, the reported values were 0.014 mM and 0.307 s^{-1}, respectively. Hence, further studies on this enzyme have moved towards understanding its structure–function relationship and improving its catalytic efficiency in converting penicillin G.

DAOCS: a Fe(II) and 2-oxoglutarate dependent dioxygenase

DAOCS belongs to a family of enzymes that utilizes an iron(II) ion as a co-factor and 2-oxoglutarate as a co-substrate to catalyse oxidative reactions. The enzymes in this

Table 2 *Streptomyces clavuligerus* DAOCS has wide substrate specificity



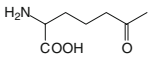
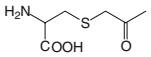
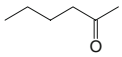
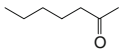
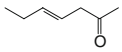
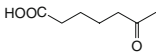
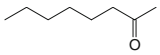
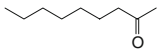
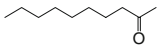
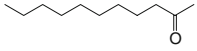
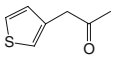
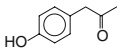
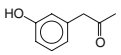
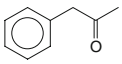
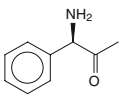
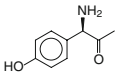
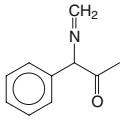
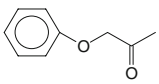
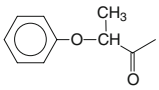
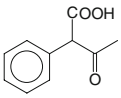
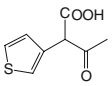
Penicillin substrate	Side chain, R	References
Penicillin N		[60]
D-Carboxymethylcysteinyl-6-APA		[60]
Acetyl-6-APA		[29]
Butyryl-6-APA		[18]
Valeryl-6-APA		[18]
Hexanoyl-6-APA		[18]
Penicillin F		[18]
Adipyl-6-APA		[18]
Heptanoyl-6-APA		[18]
Octanoyl-6-APA		[18]
Nonanoyl-6-APA		[18]
Decanoyl-6-APA		[18]
2-Thiophenylacetyl-6-APA		[18]
Penicillin X		[18]
Penicillin mX		[18]
Penicillin G		[17, 18, 29, 80]
Ampicillin		[17, 18, 29, 75, 80]

Table 2 continued

Penicillin substrate	Side chain, R	References
Amoxicillin		[17]
Metampicillin		[17]
Penicillin V		[17, 18]
Phenethicillin		[17]
Carbenicillin		[17]
Ticarcillin		Unpublished

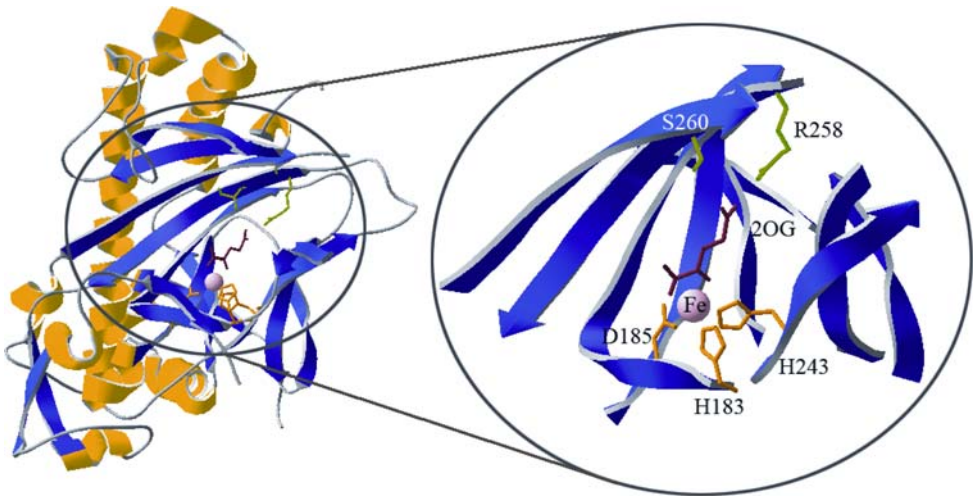


Fig. 2 Crystal structure of scDAOCS (PDB: 1UNB) depicting the characteristic β-barrel and conserved *HXD/EX$_n$H* and *RXS* motifs in the Fe(II)/2-oxoglutarate-dependent dioxygenase enzyme family. Residues H183, D185 and H243 are involved in binding Fe(II) ion whereas S260 and R258 stabilize the binding of 2-oxoglutarate (2OG) through hydrogen-bonding interactions (*not shown*)

family have a characteristic "jelly-roll" structure made up of eight β-sheets, with four β-sheets on each side of the barrel (Fig. 2). The β-barrel accommodates the conserved *HXD/EX$_n$H* and *RXS* motifs essential for iron(II) ion and 2-oxoglutarate binding, respectively [41]; in the case of DAOCS, they are H183, D185, H243, R258, and S260 [79, 81]. Other antibiotic-producing enzymes in this family, for example clavaminate synthase (CAS) and carbapenem synthase, also have similar structural features [21, 59].

The iron-binding centre in this family of enzymes essentially provides versatility and flexibility in catalysing a variety of oxidative reactions that include desaturation, ring expansion, and hydroxylation. These are achieved by generation of a reactive iron species that is required to attack the substrates. The resulting oxidative reaction would depend on the internal architecture of the enzyme where the spatial arrangements of the substrate relative to the activated iron centre are important considerations for eliciting reactions. The sequence of events from iron(II) ion and 2-oxoglutarate binding to substrate oxidation may also differ slightly between enzymes, for similar reasons. For example, in CAS, simultaneous 2-oxoglutarate and

Table 3 Crystal structures of DAOCS deposited in the PDB[a]

Enzyme	Crystallized form	Resolution (Å)	PDB[a]	References
scDAOCS	Apoprotein	1.3	1DCS	[85]
	Fe(II)	1.5	1RXF	
	Fe(II), 2-oxoglutarate	1.5	1RXG	
scDAOCS[b]				
R258Q	Fe(II), α-keto-β-methylbutanoate	1.5	1HJG	[52]
R258Q	Fe(II), α-keto-β-methylpentanoate	1.6	1HJF	
scDAOCS[3]				
ΔR306	Fe(II), 2-oxoglutarate	2.1	1E5I	[53]
ΔR307A	Fe(II), succinate, CO_2	1.96	1E5H	
scDAOCS	Fe(II), succinate	1.5	1UO9	[84]
	Fe(II), penicillin G	1.6	1UOF	
	Fe(II), deacetoxycephalosporin C	1.7	1UOG	
	Fe(II), 2-oxoglutarate, penicillin G	1.7	1UOB	
	Fe(II), 2-oxoglutarate, ampicillin	1.5	1UNB	
scDAOCS[c]	Apoprotein	2.3	1W28	[67]
	Fe(II)	2.51	1W2A	
	Fe(II), ampicillin	2.7	1W2 N	
	Fe(II), deacetoxycephalosporin C	3.0	1W2O	
scDAOCS	5-hydroxy-4-ketovaleric acid	1.53	2JB8	[19]

The crystal structures are arranged in chronological order

[a] PDB accession number

[b] scDAOCS with mutational alteration

[c] scDAOCS with N-terminal His-tag

substrate binding are necessary to activate the iron centre whereas in DAOCS, binding of 2-oxoglutarate and penicillin substrates are mutually exclusive, as supported by structural studies described below [84, 94].

Structural and mechanistic studies of DAOCS

To date, a total of 17 crystal structures of scDAOCS have been reported and deposited in the Protein Data Bank (PDB) (Table 3). The first structure published by Valegård et al. [85] provided an important view of the tertiary folding of the enzyme and physical evidence of residues involved in the iron centre and interaction with the co-substrate. The study revealed that scDAOCS apoenzyme exists as a trimeric unit, where the C-terminus of one molecule (residues 308–311) penetrates the active site of its neighbouring molecule in a cyclical fashion. However, upon addition of Fe(II) and 2-oxoglutarate, scDAOCS dissociates into monomers. These observations were supported by gel filtration and dynamic laser light scattering studies which revealed the existence of an equilibrium between the monomeric and trimeric scDAOCS in the absence of iron(II), and a shift towards the monomeric form upon addition of the co-factor [57]. These findings implied that monomeric scDAOCS is the catalytically active form facilitating the oxidative reaction.

The lack of a substrate in the crystal structure has, then, limited understanding on the catalytic mechanism of scDAOCS. The stable native trimeric structure of scDAOCS made crystallization of an enzyme–substrate complex difficult, as the active site is partially occupied by the C-terminal tail of the neighbouring enzyme. Truncation of the C-terminal tail helped to prevent trimer formation but the method resulted in a loss of enzymatic activity. Oster et al. [67] reported that attachment of a His-tag at the N-terminus of scDAOCS disrupts trimer formation and liberates the C-terminal arm of the apoenzyme. However, analyses of the biochemical and structural data revealed significant changes in the substrate-binding affinity and orientation of the penicillin nucleus in the His-tagged scDAOCS–substrate complex structure compared with those in the native enzyme–substrate structure published by the same group [84]. These discrepancies suggest caution when selecting the appropriate structural template for subsequent studies. More importantly, the data illustrated that the activity of scDAOCS very much depends on the stability of the enzyme maintained by equilibrium in a pool of monomeric and trimeric forms of scDAOCS which can be disrupted by introduction of a relatively small His-tag at the N-terminus of the enzyme. Nevertheless, in both types of structure, the C-terminus remained disordered in the enzyme–substrate complex, implying a degree of flexibility that may be crucial for catalytic activity, as supported by mutagenesis studies [16, 17, 53, 54].

The elucidation of scDAOCS structures with iron(II), 2-oxoglutarate, and penicillins was instrumental in forming the current "booby-trap" mechanism hypothesis [84]. Several important findings from the structural studies are worth mentioning:

(1) The penicillin substrates and 2-oxoglutarate share overlapping binding sites, implying mutual exclusivity

in binding and hence the requirement of an ordered sequential binding for catalysis; and

(2) The orientation of penicillin G differs in DAOCS complexes formed with or without the addition of 2-oxoglutarate, with the former resembling the orientation of product DAOC co-crystallized with DAOCS. This suggests that 2-oxoglutarate is necessary to influence the orientation of penicillin binding and, thus, productive ring-expansion of penicillin.

On the basis of these findings, the current postulated mechanism involves initial activation of the iron centre by 2-oxoglutarate binding followed by oxidative decarboxylation of 2-oxoglutarate to generate oxidizing ferryl species with succinate and carbon dioxide as by-products. Succinate remains bound and stabilizes the reactive iron species until the substrate enters the active site. Binding of penicillin to the reactive iron species via the sulfur group subsequently expels succinate and leads to oxidative ring expansion of the substrate.

Apart from providing insights to the mechanism of catalysis, the crystal structures of DAOCS with penicillins have also served as platforms to identify important residues that interact with the substrate. Some of these residues have the potential to improve the enzymatic activity of DAOCS by mutational alteration. The efforts in improving DAOCS activity for industrial applications are described in the subsequent sections of this review.

Alteration of deacetoxycephalosporin C synthase co-substrate selectivity

Elucidation of the tertiary structure of a protein is essential for understanding the mechanism of substrate recognition and catalysis. Henceforth, it will be easier to rationally modify the enzyme substrate preference to suit industrial needs. This has been illustrated by successful alteration of the co-substrate selectivity of scDAOCS [52]. The crystal structure of scDAOCS complexed with 2-oxoglutarate revealed that the 5-carboxylate of 2-oxoglutarate is stabilized by electrostatic interaction with the side chain of R258 [85]. Lee et al. [52] showed that R258Q mutation in scDAOCS almost abolished the oxidation of both penicillin N and penicillin G. Interestingly, aliphatic 2-oxoacids (2-oxo-4-methylpentanoate and 2-oxo-3-methylbutanoate), which could not replace the function of 2-oxoglutarate for the wild-type enzyme, were able to "rescue" the catalytic activity of the R258Q mutant to the level observed for the wild-type enzyme. Crystallographic studies of this mutant suggested that the binding of the other aliphatic 2-oxoacids was similar to that of 2-oxoglutarate except for the electrostatic interaction. This indicated the importance of this amino acid position in determining the co-substrate selectivity of scDAOCS. This is further supported by another study in which R258L mutant was demonstrated to have the same catalytic activity as the R258Q mutant when 2-oxo-4-methylpentanoate was used as a co-substrate [51]. Hence, by modifying the critical co-substrate binding residue, it is possible to alter the co-substrate preference of scDAOCS without compromising its ring-expansion activity.

Enzyme modification to improve the substrate binding and catalytic activity of DAOCS

Various mutational studies aimed at understanding the molecular mechanism of DAOCS activity have revealed certain amino acid residues that are important for catalysis and others that are amenable to modification to improve the functions of the enzyme. Generally, the approach to enzyme improvement can be divided into two main strategies:

(1) the directed evolution approach, which can be performed without prior knowledge of the enzyme tertiary structure but often requires a large recombinant library to increase the chance of obtaining a clone with the desired property; and

(2) the rational approach, in which knowledge of its tertiary structure is necessary to engineer modification of the enzyme to achieve the desired properties.

Nevertheless, both approaches have generated important information with regard to the structure–function relationship of scDAOCS.

Directed evolution approach

The directed evolution approach mimics the evolutionary process of proteins by exploiting random mutagenesis techniques, for example homologous and non-homologous recombinations, error-prone PCR, and chemical and physical mutagenesis methods, coupled with an efficient screening or selection method to obtain proteins with the desired properties. The first law of this approach, "you get what you screen for", always applies. Hence, the screening method is an important part of the whole experiment. The design of the screening method has to ensure efficient detection of product, which is a reflection of the desired property of the protein, and yet, the outcome is not guaranteed. Often, a large library of recombinants is required and the number of desired recombinants could be very low. Nevertheless, this approach has also provided some important information on the structure–function relationship of DAOCS.

Homologous recombination

The first reported attempt to improve the activity of DAOCS by the directed evolution approach was probably the construction of hybrid DAOCS of *S. clavuligerus* and *N. lactamdurans* by Adrio et al. using in-vivo homologous recombination [2, 4]. This technique exploited the advantage of natural recombination at homologous regions of closely related sequences in a host organism to generate a recombinant enzyme. Rapid visual screening was set up exploiting a tyrosinase *mel*C gene from *S. glaucescens*, which encodes the ability to secrete the black pigment melanin. The *mel*C gene was inserted between the two *cef*E genes in a pJA680 vector and recombination of the two *cef*E genes would result in loss of pigment production by the host organism. Using *S. lividans* strain 1326, a recombinant strain W25 was generated and found to have higher penicillin G-conversion activity than the *S. clavuligerus* NP1 strain [2, 4]. Further analyses revealed that this recombinant strain preferred a lower reaction temperature (<30°C), a lower iron(II) concentration, and a higher level of 2-oxoglutarate for penicillin G conversion than *S. clavuligerus* NP1 strain [33].

A similar method was adopted by Hsu et al. [42] to improve the substrate specificity of DAOCS for penicillin G, except that it is an in-vitro recombination method and more than two gene sequences were used for shuffling. The *cef*E genes from *S. clavuligerus* (ATCC 27064), *N. lactamdurans* (ATCC 27382), *S. jumonjinensis* (ATCC 29864), *S. ambofaciens* (102SH3), and *S. chartreusis* (29SA4), the *cef*F gene from *S. clavuligerus*, which encodes for deacetylcephalosporin C synthase, and the *cef*EF gene from *A. chrysogenum* (ATCC 11550), were used for the shuffling experiment. These shuffled genes were heterologously expressed in *E. coli* cells and the transformants were screened for penicillin G conversion activity. After two rounds of gene shuffling and screening, two clones, FF1 and FF8, showed no substrate inhibition and were subjected to kinetic and sequence analyses. FF1 has an increment in k_{cat}/K_m value of approximately ninefold when compared with scDAOCS, and FF8 has a 118-fold increment, which is probably the highest improvement recorded so far for penicillin G conversion. Comparison of the amino acid sequence of FF8 with that of scDAOCS revealed that only two of the 35 substitutions, i.e., residues M73 and I305, are at positions known to have direct interactions with the substrate.

Chemical mutagenesis

Chemical mutagenesis using hydroxylamine was attempted by Wei et al. [90] with the objective of modifying scDAOCS for optimum ring expansion activity towards penicillin G. The mutant library was first screened by a simple high throughput paper disk-based bioassay followed by a second screening method using thin-layer chromatography with radio-labeled penicillin G as a substrate. From 5,500 clones screened, six were found to have increased conversion activity and these were because of three point mutations, G79E, V275I, and C281Y. Because these three sites are located away from the substrate binding pocket, the improvement achieved could have been because of a global effect of these mutations on the conformation of the active site [90].

Error-prone PCR combined with gene shuffling

Error-prone PCR exploits the altered fidelity of DNA polymerase in the presence of manganese ions to incorporate mismatch nucleotides during PCR amplification of the gene of interest. Wei et al. [89] used a ratio of Mg(II) ions to Mn(II) ions of 12:1 in the error-prone PCR process to induce mutations. Approximately 6,000 clones were subjected to a screening process and six types of point mutations (M73T, T91A, M188V, M188I, H244Q, G300V) and six types of double mutations (D53H/C281Y, M73T/P145L, A106T/C155Y, A106T/M188V, D107G/L277Q, Y184H/C281R) were identified. Subsequently, single mutations based on the double mutations were constructed to assess the effect of each substitution. Among these mutations, the L277Q mutant had the highest catalytic efficiency for penicillin G (sixfold increment), whereas the M73T, G79E, T91A, Y184H, M188V, and H244Q mutants had approximately two to fivefold increment in k_{cat}/K_m values when compared with the wild-type enzyme. Other mutations either had activities similar to that of the wild-type enzyme or exhibited substrate inhibition. Gene shuffling of these single mutants, including those reported previously, i.e., G79E, V275I, C281Y, N304K, I305L, and I305M mutants [90], was then conducted to generate random combinations. Approximately 10,000 clones were screened and only one quaternary mutant (C155Y/Y184H/V275I/C281Y) with enhanced activity and without substrate inhibition was subjected to kinetic analysis. For this mutant the k_{cat}/K_m value for penicillin G conversion was a further sevenfold higher than for the C281Y single mutant, which had the highest k_{cat}/K_m value among these four single mutants (sixfold higher than for the wild-type enzyme). Table 4 summarizes the kinetic data for selected examples of mutant scDAOCSs with improved activities obtained by Wei et al. [89, 90] and Hsu et al. [42].

Comparison of random mutagenesis methods

Comparing these techniques, hydroxylamine mutagenesis generated the least number of mutants with improved penicillin G conversion activities. This chemical targets mainly

Table 4 Kinetic data for penicillin G conversion by bioengineered DAOCSs [42, 89, 90]

Mutations	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	Relative k_{cat}/K_m
Wild-type	2.58	0.0453	18	1
M73T	0.74	0.0627	85	4.7
G79E	0.75	0.0315	42	2.3
T91A	1.36	0.0522	38	2.1
C155Y	1.76	0.0476	27	1.5
Y184H	0.95	0.0798	84	4.7
M188 V	0.76	0.0456	60	3.3
H244Q	1.39	0.0698	50	2.8
V275I	1.68	0.0502	30	1.7
L277Q	1.02	0.1036	102	5.7
C281Y	0.68	0.0744	109	6.1
N304 K	0.22	0.0564	256	14.2
I305L	0.66	0.0759	115	6.4
I305 M	0.75	0.1452	194	10.8
V275I/I305 M	0.25	0.1458	583	32.4
C155Y/Y184H/V275I/C281Y	0.19	0.1398	736	40.9
FF8[a]	0.014	0.0297	2121	117.8

[a] Recombinant derived from gene shuffling [42]

the cytosine residues in DNA which limits the repertoire of mutations [83]. In contrast, error-prone PCR generated a reasonably large number of mutants with improved activities. However, this method has errors biassed toward AT to GC changes [20]. Furthermore, codon redundancy and possible introduction of stop codons lower the efficiency of this method in generating desired mutations. Gene shuffling has been successfully employed in generating an efficient recombinant DAOCS with comparable catalytic efficiency in penicillin G conversion with that of scDAOCS in penicillin N conversion. Although it is still not clear which amino acid differences between this recombinant and scDAOCS are responsible for this substantial improvement, this technique is a promising route in generating recombinants with desired substrate specificity, especially when more DAOCS orthologues are available for shuffling.

Together, these studies suggest that the substrate specificity of DAOCS is not solely governed by the substrate-binding residues. Amino acid residues outside the substrate-binding site may also affect the substrate specificity of the enzyme and these residues are often difficult to identify by the rational approach (Fig. 3). However, the effects of the mutations identified cannot be rationalized without structural information about the enzyme. In order to understand the molecular basis of such mutations in influencing the function of the enzyme, further analyses requiring the rational approach must be performed.

Rational approach

The three-dimensional structure of the DAOCS-Fe(II)-2-oxoglutarate complex was resolved in the late 1990s [57, 85] but the lack of bound substrate hindered many subsequent studies. It was recognized that the crystal structure of DAOCS resembles that of IPNS [69, 85]. The crystal structure of the DAOCS–Fe(II)–2-oxoglutarate complex was superimposed on that of the IPNS–Fe(II)–ACV complex to predict the possible substrate binding pocket of scDAOCS [16, 85]. Residues R74, R160, R266, and N304

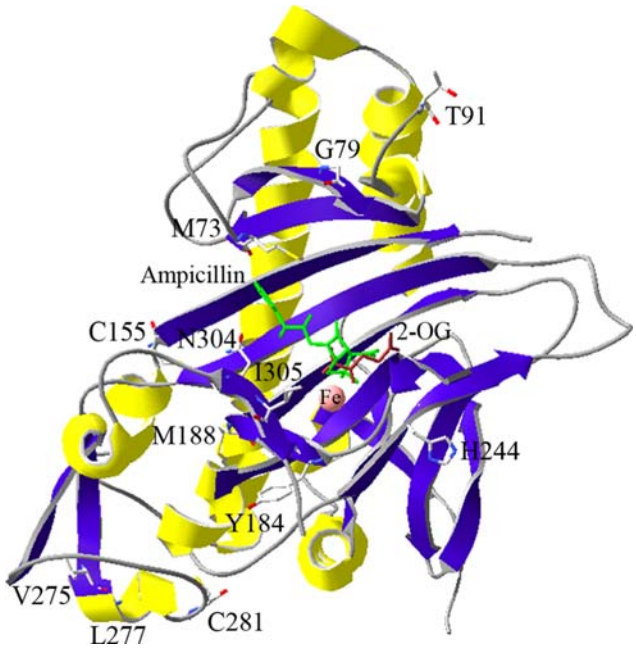


Fig. 3 Spatial locations of useful mutations in scDAOCS (PDB: 1UNB) identified by using directed evolution approaches. Among the mutations listed in Table 4, only M73T, N304K, and I305M/L mutations are localized in the substrate-binding site with the side chains of the amino acid residues interacting with the penicillin substrate

were selected for site-directed mutagenesis studies based on the close proximity to the L-α-aminoadipyl branch of ACV and their hydropathic properties [16]. This study showed that only the N304L mutation increased its penicillin analog conversion activity. The R74L, R160L, and R266L mutations abolished the activity of scDAOCS and this indicated the importance of these residues in binding the penicillin substrate. Similarly, Lee et al. [54] also showed that the N304A mutation was able to increase the activity of the enzyme. Thereafter, Wei et al. [90] also adopted the same approach and reported that the N304K, I305L, and I305M mutations were able to increase the activity of scDAOCS (Table 4). Although results from various structural studies suggested that the C-terminal of scDAOCS (residues 301–311) might be involved in guiding the substrate into the substrate binding pocket [53], the rationale behind these mutations could not be clearly explained in the absence of a crystal structure of DAOCS complexed with a penicillin substrate.

Complete library of amino acid replacements

In order to elucidate the molecular basis of substrate interaction by residue N304, Chin et al. [14] performed a study using a complete library of amino acid alterations and found that mutation to basic residues (arginine and lysine) has better effects than mutation to aliphatic residues (alanine, leucine, and methionine) in the conversion of seven hydrophobic penicillins. Supported by the resolution of a crystal structure of scDAOCS complex with penicillin G and ampicillin, it was realized that the side chain of asparagine at position 304 aligns with that of the penicillin substrate [84]. Hence, the observed improvement is probably because of better hydrophobic interaction between the side chains of arginine and lysine and that of the penicillin substrate, leading to a lower K_m value [37].

Mutation of the residue R306 to leucine has also been demonstrated to improve the conversion activity of scDAOCS [17]. The crystal structure revealed that the guanidinium side chain of residue R306 is located away from the catalytic site and interacts with a hydrophobic cleft formed by residues Y184, L186, W297, I298, and V303. It was clear that the improved conversion activity conferred by R306L mutation was not because of direct interaction with the substrate. In order to further understand the role of R306 in the conversion activity of scDAOCS, a similar approach to the N304 mutation studies was adopted [36]. From this study it was deduced that the length and hydrophobicity of the amino acid side chain at amino acid position 306 are important in influencing the function of the enzyme. The loss of enzyme activity by R306P mutation indicated that the conformation of the C-terminal of scDAOCS is crucial for its catalytic property. Replacement with charged residues resulted in a tremendous decrease in the conversion activity of scDAOCS, which retained less than 20% of the wild-type activity for all substrates. This supported the postulation that charged residues are detrimental to this site, probably because they perturb the integrity of the hydrophobic cleft. Although both arginine and lysine residues are positively-charged amino acids, the pK_a of their side chains would be reduced if they were buried in a hydrophobic environment [38]. This would render the amino groups in the side chains of both residues unprotonated and uncharged. Consequently, the integrity at this region might be preserved. Replacement of R306 with amino acids structurally similar to leucine, i.e., isoleucine and methionine, was able to improve the enzyme activity via hydrophobic interaction with the surrounding residues. As reflected by their K_m values for ampicillin conversion, the improvements were obtained because of enhancement of the substrate-binding affinity of the enzyme by these mutations [36].

Although this approach of using a complete library of amino acid replacement mutants is laborious and time-consuming, the effects of all possible mutations at that position of the protein will be determined and the ideal amino acid residue that improves the function of the enzyme will be revealed. This method will be better when it is coupled with structural studies to elucidate the molecular mechanism behind the improvement.

Double mutation analysis

Generally, more than one mutation of the enzyme is required to alter its property towards a desirable one. When two or more mutations are combined in a single protein, there could be various effects [64, 91]. Therefore, it is necessary to critically assess the effects of each combination on its own merits. Hence, various combinations of double mutation based on V275I, C281Y, and C-terminal mutations were generated and evaluated [37]. Kinetic analyses using ampicillin as a substrate showed that the k_{cat}/K_m values of all double-mutant scDAOCSs were higher than that of the wild-type enzyme for ampicillin conversion. Two double mutants, C281Y/N304R and C281Y/I305M, showed the greatest improvement in the enzyme's catalytic efficiency (101 and 68-fold increments, respectively). This study also suggested that the N304K and N304R mutations are ideal for improving the substrate-binding affinity of the enzyme whereas the I305M mutation is ideal for increasing the turnover rate [37].

Future directions of enzyme improvement in DAOCS

It has been about 30 years since the discovery of ring-expansion activity in cephalosporin-producing microorganisms.

Advances in the techniques of molecular biology in the last few decades have propelled research of DAOCS from purifying the native enzyme to heterologous production of recombinant enzyme in various hosts for biochemical characterization. With the "green chemistry" movement, DAOCS has a relevant application in the pharmaceutical industries to produce existing, and novel and more efficacious, cephalosporins. Although early studies on DAOCS identified it as having narrow substrate specificity, relentless efforts have shown otherwise. Structural studies have provided important information about its folding and catalytic mechanism, and protein engineering has enabled modification of DAOCS to improve its function to suit industrial needs. However, the catalytic efficiency of these modified DAOCSs has yet to match that of the wild-type enzyme in its natural substrate conversion.

There are numerous productive modifications of substrate specificity of different enzymes using directed evolution methods, but knowledge from these studies may not be universally applied to all proteins [5]. Nonetheless, the techniques are invaluable tools for improving enzyme functions and some have helped to identify relevant sites in DAOCS for engineering its substrate specificity. Application of different mutagenic techniques developed to overcome the limitations of any individual technique extends the possibility of obtaining a DAOCS with improved catalytic properties. However, the screening of the recombinant libraries is a tedious process and progress in this area would be greatly facilitated by the development of high-throughput screening techniques or selective methods for identifying clones with the desired properties.

Many studies on the substrate specificity of DAOCS have been focussed on penicillin G, because it is a readily available, inexpensive source of substrate for the industrial production of cephalosporin. Penicillin V can be exploited as an alternative substrate, because it also is commercially available. However, its potential application has yet to be explored. Studies using bioassays have shown some promising results—the relative specific activities of the mutant scDAOCSs were higher than that of the wild-type enzyme in penicillin V conversion [14, 17]. However, to consider its industrial application, quantitative analysis of penicillin V conversion relative to that of penicillin G would be necessary. It will also be interesting to investigate how the structural difference between these two substrates leads to a performance disparity in the wild-type scDAOCS, and this could potentially provide further insights into modification of scDAOCS to accept various types of penicillin.

In the prokaryotic β-lactam biosynthetic pathway, the step after deacetoxycephalosporin synthesis involves the hydroxylation of deacetoxycephalosporin to a deacetylcephalosporin, which is executed by deacetylcephalosporin C synthase (DACS). DACS has approximately 74% primary protein sequence similarity to DAOCS, suggesting a gene duplication event during evolution that led to two homologous enzymes catalysing different reactions [6]. However, it is still not clear how two highly conserved enzymes are able to drive two types of reaction using the same catalytic platform. Although a mutagenic study on fungal DAOC/DACS has identified two amino acid positions that are crucial for hydroxylation [58], a similar effect has not been demonstrated in prokaryotic DACS. Further investigation of DACS hydroxylation activity should provide more insights to the structure–function relationships of DAOCS and DACS. Thus, one could effectively improve useful enzymes per se or consider synergistic approaches to making multi-functional enzymes.

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