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Mutational Analysis of Tyrosine-191 in the Catalysis of *Cephalosporium acremonium* Isopenicillin N Synthase[1]

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Isopenicillin N synthase (IPNS) is a key enzyme responsible for the catalytic conversion of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N in the β-lactam antibiotic biosynthetic pathway. The *Aspergillus nidulans* IPNS crystal structure implicated amino acid residues tyrosine-189, arginine-279, and serine-281 in the substrate-binding of the valine carboxylate portion of ACV *via* hydrogen bonds. In previous reports, we provided mutational evidence for the critical involvement of the corresponding arginine-281 and serine-283, which constitute a conserved R-X-S motif, for the catalysis of *Cephalosporium acremonium* IPNS (cIPNS). In this study, we report the site-directed mutagenesis of the corresponding tyrosine-191 in cIPNS to four amino acids from different amino acid groups, namely, phenylalanine, serine, histidine, and aspartate. The mutants Y191F, Y191H, and Y191R respectively yielded specific activities at levels of 3, 8.6, and 18.8% relative to the wild-type when enzyme bioassays were performed using purified protein fractions. These results were surprising, as previous mutational analyses involving arginine-281 and serine-283 resulted in non-measurable specific activities, thus suggesting that tyrosine-191 is important but not critical for the activity of cIPNS due to its involvement in ACV binding. Hence, it is likely that tyrosine-191 is the least critical of the three residues involved in binding the ACV valine carboxylate moiety.

Key words: isopenicillin N synthase, site-directed mutagenesis, substrate-binding.

The research and development efforts in the field of β-lactam antibiotics continue to have an exciting outlook, even after seventy years (*1*). These antibiotics, which include the penicillins and cephalosporins, are highly favoured due to their low toxicity and high efficacy (*1*). A key step in the penicillin and cephalosporin antibiotic biosynthetic pathway involves the biotransformation of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to form a bicyclic β-lactam-thiazolidine structure called isopenicillin N (IPN) (*2*). This unique biochemical reaction, which utilises the full oxidising potential of oxygen, is catalysed by isopenicillin N synthase (IPNS), a non-heme iron oxidase (*3*).

Recent work on IPNS involving elucidation of *Aspergillus nidulans* IPNS (aIPNS) crystal structures (*4*, *5*) and site-directed mutagenesis experiments with *Cephalosporium acremonium* and *Streptomyces jumonjinensis* IPNSs (*6–8*) has established that a catalytic motif, HisXAsp(53-57)XHis, which encompasses the amino acids histidine-214, aspartate-216, and histidine-270 (aIPNS numbering), is critical for catalysis. Amino acid residues implicated in the substrate-binding of ACV include arginine-87, tyrosine-189, arginine-279, and serine-281 (aIPNS numbering) (*5*). Arginine-87 is involved in the binding of the aminoadipoyl carboxylate portion of ACV *via* the formation of a salt bridge, whereas tyrosine-189, arginine-279, and serine-281 are involved in the binding of the valine carboxylate portion of ACV *via* hydrogen bonds.

Arginine-279 and serine-281 constitute a conserved R-X-S motif which is prevalent among related members of the non-heme iron dioxygenase family (*9*). This motif was implicated in the activity of the related flavanone 3β-hydroxylases, by providing a permanent positive charge for the binding of the cosubstrate 2-oxoglutarate (*9*). Similarly, in the related 1-aminocyclopropane-1-carboxylate oxidases (ACCO), this motif was suggested to bind to the carboxylate portion of the substrate 1-aminocyclopropane-1-carboxylate (*3*). We have earlier provided mutational evidence in support of the aIPNS crystal structure for the role of this particular R-X-S motif (the corresponding arginine-281 and serine-283) in ACV binding in *C. acremonium* IPNS (cIPNS) (*10*, *11*). As the crystal structure also implicated a third residue, tyrosine-189, in association with the conserved R-X-S motif, in binding the valine carboxylate portion of ACV, it would be interesting to investigate the effect on IPNS catalysis when this residue is replaced with different amino acids.

A multiple sequence alignment of aIPNS and other related non-heme iron oxidases showed that this particular tyrosine-189 corresponded to a lysine-158 in ACCO (*12*). Site-specific alteration of this lysine residue in kiwi fruit ACCO resulted in the retention of a relative specific activity of 1%, although no clear role in catalysis was assigned to this residue (*12*). Although a lysine residue was thought to be critical for carbon dioxide activation in ACCOs (*13*), this particular lysine residue was instead proposed to play

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a structural role in the maintenance of protein structure by the authors.

In this study, we performed site-directed mutagenesis on the corresponding tyrosine-191 in cIPNS to assess the effects of amino acid substitutions on enzyme catalysis. Four different amino acids, phenylalanine, serine, histidine, and arginine, were introduced, and their ability to perform the role of tyrosine-191 was investigated. Phenylalanine was chosen as it has the closest side-chain structure to tyrosine with the exception of the absent hydroxyl group. Serine possesses the hydroxyl group but lacks the bulky benzene ring. Structurally, this represents the most drastic side-chain substitution. Histidine replaces the side-chain of tyrosine with an imidazole group, and arginine introduces a basic guanidino side chain.

The cIPNS gene was amplified by the polymerase chain reaction (PCR) and subcloned into a modified kanamycin-derived glutathione S-transferase (GST) fusion vector, where GST is the fusion partner. In the course of this study, we found that glutathione does not seem to inhibit *in vitro* IPNS activity, contrary to the suggestion of Ramos *et al.* (1985) that glutathione may act as a competitive inhibitor (*14*).

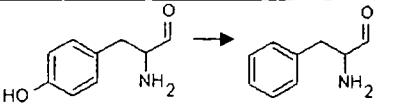
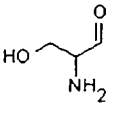
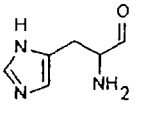
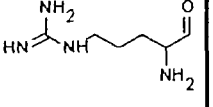
MATERIAL AND METHODS

Construction of pGK, a Kanamycin-Resistance Derivative of pGEX-6P-1—The ampicillin resistance gene (β-lactamase) from the pGEX-6P-1 vector was removed using the restriction enzymes *Xho*I and *Alw*NI, as the expression of β-lactamase would interfere with IPNS enzymatic activity. Using the same restriction enzymes, the kanamycin resistance gene was removed from the pET24a vector (Novagen) and ligated into the pGEX-6P-1 vector devoid of β-lactamase. This modified fusion vector which confers kanamycin resistance to the host cell was termed pGK and was used for subsequent experiments.

PCR Cloning of cIPNS into pGK—The primers OL183/F 5′ ACC***GGATCC***ATGGGTTCCGTTCCAGTTCC 3′ (*Bam*HI restriction enzyme site in bold and italics) and OL 184/R 5′ GC***GAATTC***ACCTGATCGACCGATTTAGG 3′ (*Eco*RI restriction enzyme site in bold and italics) were used to amplify the cIPNS gene from the recombinant vector pXW523 (*15*) harbouring the wild-type cIPNS gene. PCR was performed in a 50 μl reaction mixture [1 x Perkin-Elmer® Buffer II, 1.65 mM $MgCl_2$, 6% dimethyl sulfoxide (DMSO), 200 μM deoxyribo-nucleoside triphosphate (dNTP), 10 pmol/μl of each primer, 3 U AmpliTaq Gold™ DNA polymerase, and approximately 500 ng of pXW523] using the following touch-down amplification profile: 8 cycles of 95°C (30 s), 60–56.5°C (30 s), 72°C (1 min), followed by 27 cycles of 95°C (30 s), 60°C (30 s), 72°C (1 min), and a final extension step at 72°C for 5 min. A negative control was performed under the same conditions without the addition of vector DNA.

The PCR product was subjected to agarose gel electrophoresis using a 0.8% agarose gel to confirm successful amplification. The cIPNS PCR product was then ligated at 4°C according to the manufacturer's instructions to the pGEM®-T Easy vector (Promega), which has single 3′-T overhangs for the efficient ligation of PCR products. The ligation mix was subsequently transformed into *Escherichia coli* DH5α, and recombinant plasmid constructs of pGEM®-T Easy containing the cIPNS gene were extracted using the Wizard® *Plus* Minipreps DNA Purification System (Promega) and sequenced on both strands using the ABI PRISM™ Big-Dye™ terminator cycle sequencing kit (PE Applied Biosystems). Utilizing the restriction enzymes *Bam*HI (Strata-

TABLE I. **Description of the mutagenic primer pairs and amino acid replacements in this study.**

Amino acid change	Mutagenic primer sequence (base change in bold and italics)	Description of "changed" amino acid (from tyrosine, with a polar uncharged phenol side chain)	Diagrammatic alteration of the amino acid side-chain
Tyrosine → Phenylalanine (Y→F)	OL193/F, *5′ TG CTC ATC CGT* ***TT****C CCG TAC CTC G 3′* and OL194/R, *5′ C GAG GTA CGG* ***GA****A ACG GAT GAG CA 3′*	Non-polar phenyl side chain	
Tyrosine → Serine (Y→S)	OL270/F, *5′ GTG CTC ATC CGT* ***TC****C CCG TAC CTC GAC 3′* and OL271/R, *5′ GTC GAG GTA CGG* ***GG****A ACG GAT GAG CAC 3′*	Uncharged, hydrophilic polar amino acid with a hydroxyl group	
Tyrosine → Histidine (Y→ H)	OL319/F, *5′ GTG CTC ATC CGT* ***CA****C CCG TAC CTC GAC 3′* and OL320/R, *5′ GTC GAG GTA CGG* ***GTG*** *ACG GAT GAG CAC 3′*	Imidazole side chain amino acid	
Tyrosine → Arginine (Y→R)	OL321/F, *5′ GTG CTC ATC CGT* ***CG****C CCG TAC CTC GAC 3′* and OL322/R, *5′ GTC GAG GTA CGG* ***GCG*** *ACG GAT GAG CAC 3′*	Basic guanidino amino acid	

gene) and *Eco*RI (Promega), the cIPNS gene was extracted from the recombinant pGEM®-T Easy constructs and subcloned into pGK, the modified kanamycin-derived GST fusion vector. The recombinant fusion construct, termed pGKC, was transformed into *E. coli* BL21-(DE3) for propagation and heterologous expression.

Site-Directed Mutagenesis and Sequencing—For *in vitro* site-directed mutagenesis, the Quik-change site-directed mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. The number of polymerisation cycles ranges from 20 to 25 for our purpose. Each mutant gene was sequenced in its entirety to confirm the occurrence of the site-specific mutation. Mutagenic primer pairs used for the replacement of tyrosine-191 and the different side-chain structures of the amino acids investigated in this study are tabulated in Table I.

Heterologous Expression and Protein Purification, IPNS Enzymatic Assays, Protein Determination and Scanning Densitometry—Expression and enzymatic assays of the wild-type and mutant cIPNSs were carried out as previously described (*6*). Glutathione was added to an IPNS bioassay to investigate the effect of this cofactor. Expression was carried out for 15 h post-induction at 37°C, or at 25°C when a soluble expression product was not obtainable at 37°C. Protein purification was performed using Glutathione Sepharose 4B for binding the GST-cIPNS fusion protein according to the manufacturer's instructions. Subsequently, an appropriate amount of PreScission™ Protease was added to cleave the cIPNS protein from its GST fusion partner by incubation at 4°C for 5 h.

Protein was quantified using the Protein Assay Reagent (Bio-Rad) according to the manufacturer's instructions. Relative amounts of expressed protein in the soluble form were determined by scanning densitometry using the Bio-Rad GS-700 Imaging Densitometer.

Computer Analysis of Wild-type and Mutant cIPNS—Protein secondary structure predictions were performed using the PEPTIDE STRUCTURE and PLOT STRUCTURE programmes based on the Chou-Fasman algorithm (*15*). The Cn3D version 2.5 helper application for viewing three-di-mensional structures was obtained from the National Center for Biotechnology Information's website. The accession numbers for the aIPNS crystal structure are 1BK0 and 1BKZ. Virtual mutagenesis, simple modelling, further manipulation and viewing of the 3-D structure were performed using the Swiss-PdbViewer version 3.5 downloaded from the Expasy Molecular Biology server (*16*, *17*).

RESULTS

Expression and Purification of the Wild-type and Mutant cIPNSs—Expression of the recombinant GST fusion vector pGKC containing the wild-type cIPNS gene was performed initially at 37°C for 15 h post-induction, and soluble expression of up to 19% of total soluble protein was obtained [sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) shown in Fig. 1]. Similarly, soluble expression of the mutants Y191F, Y191S, and Y191H was achieved at 37°C, whereas for the mutant Y191R, post-induction temperature was reduced to 25°C to obtain soluble mutant protein. The wild-type and mutant cIPNS fusion proteins were then purified by affinity chromatography using Glutathione Sepharose 4B and cleaved by PreScission™ Protease to obtain purified proteins (Fig. 2). Wild-type and mutant cIPNS purified proteins were the main constituent of the eluted fractions (92–98%) as judged by scanning densitometry analysis.

Determination of IPNS Specific Activity by Enzyme Bioassays—The purified protein fractions of the wild-type and cIPNS mutants were assayed for enzymatic activity. Relative to the wild-type enzyme, the mutants Y191F, Y191H, and Y191R exhibited specific activity of 3, 8.6, and 18.8%, respectively (Table II). For the mutant Y191S, enzymatic activity in the purified protein fraction was not measurable, as very inconsistent small faint zones were observed, and the bioassay method itself is of limited sensitivity. The experiment was repeated with similar results. In the IPNS bioassay with soluble cell-free extracts of wild-type cIPNS, the addition of up to 10 mM glutathione did not inhibit the *in vitro* enzymatic reaction. This agrees with previous findings that glutathione does not inhibit but can mimic the action of DTT in stimulating the IPNS reaction (*18*).

Computational Analysis of the Wild-type and Mutants cIPNS—The secondary structure prediction based on the Chou-Fasman algorithm (data not shown) revealed no obvious changes in the topology of the mutants in comparison

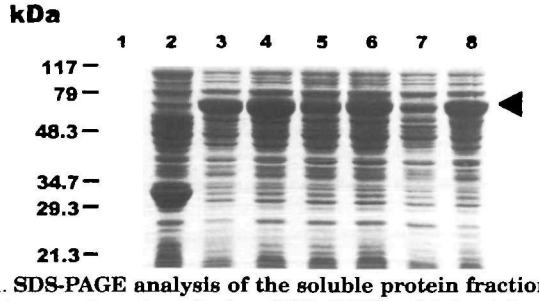


Fig. 1. **SDS-PAGE analysis of the soluble protein fractions of wild-type and mutant fusion GST-cIPNSs obtained from *E. coli* BL21(DE3) after expression at 37°C.** Lane 1, molecular mass markers; lane 2, *E. coli* BL21(DE3)/pGK vector, lane 3, wild-type GST-cIPNS; lane 4, mutant Y191F; lane 5, mutant Y191S; lane 6, mutant Y191H; lane 7, mutant Y191R; and lane 8, mutant Y191R expressed at 25°C. The arrowhead indicates the position of the fusion GST-cIPNS protein.

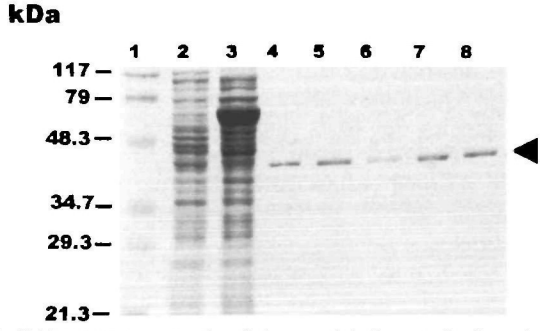


Fig. 2. **SDS-PAGE analysis of the purified protein fractions of wild-type and mutant cIPNSs.** Lane 1, molecular mass markers; lane 2, soluble protein fraction of *E. coli* BL21(DE3); lane 3, soluble protein fraction of wild-type GST-cIPNS; lane 4, wildtype cIPNS; lane 5, mutant Y191F; lane 6, mutant Y191S; lane 7, mutant Y191H; lane 8, mutant Y191R. The arrowhead indicates the position of the cIPNS protein.

TABLE II. **Activity of the purified wild-type and mutant cIPNS enzymes determined by bioassay using *Micrococcus luteus* ATCC 381 as the test organism.**

Enzyme type	Purified protein concentration (mg/ml)	Total activity (units)[a]	Specific activity (units/mg total soluble proteins)	Relative specific activity (%)	% protein purity[c]
Wild-type cIPNS	0.091	0.16	1.76	100%	98%
Y191F	0.133	0.0071	0.053	3%	95%
Y191S	0.092	n.m.[b]	n.m.	n.m.	92%
Y191H	0.127	0.0193	0.152	8.6%	96%
Y191R	0.123	0.040	0.33	18.8%	94%

[a]One unit of activity is the amount of IPNS required to form the equivalent of 1 μmol of isopenicillin N per ml per minute at 26°C. [b]Not measurable (n.m.). [c]Percentage purity determined by scanning densitometry.

with the wild-type cIPNS. Hence, based on secondary structure analyses, gross alterations of the protein conformation due to the amino acids substitutions were unlikely.

Virtual 3-D mutagenesis using the Swiss-PdbViewer was performed using the aIPNS crystal structure by altering the analogous tyrosine-189 (tyrosine-191 in cIPNS) to the various amino acids chosen for this study. Replacing tyrosine with phenylalanine eliminates the hydroxyl group and consequently the hydrogen bond between tyrosine and ACV. The other amino acid replacements also do not reconstitute any hydrogen bonds. Simple protein homology threading of cIPNS onto the aIPNS crystal structure using the SWISS-MODEL option revealed that the position of the tyrosine-191 in cIPNS would correspond to the tyrosine-189 in aIPNS.

DISCUSSION

The valine carboxylate portion of ACV interacts with tyrosine-189 (aIPNS numbering) *via* a hydrogen bond (*5*). The corresponding tyrosine-191 in cIPNS was altered to other amino acids, and the resulting mutants retain varying levels of specific activity. Thus, the possible interactions between the side-chains of these substituted amino acids and ACV might permit some suggestions on the varying differences in specific activity. In the tyrosine-to-phenylalanine replacement, the loss of the hydroxyl group eliminated the existing hydrogen bond with the valine carboxylate of ACV and thus a severe reduction of specific activity was observed. This Y191F mutation retains only 3% of the specific activity, as the benzene-ring side chain of phenylalanine is non-polar and may not be able to interact with ACV.

The replacement with serine removes the benzene ring, but the hydroxyl side-chain group in its place is likely to be too remote to interact in any way. Hence, no activity was detectable, as shown in this study. Histidine has an imidazole group which may fill the vacant slot of the phenyl group of tyrosine due to its ring structure; and because it is positively charged, it might be able to interact with the negatively charged valine carboxylate portion. To this extent, a specific activity of 8.6% is retained. Similarly, arginine's extended guanidino side-chain, which is also positively charged, may play a comparable role. The specific activity retained was greater (18.8%) with the arginine substitution than with histidine (8.6%), suggesting a stronger interaction between arginine and the valine carboxylate portion of ACV. Moreover, the basic strength and consequently the overall positive charge of arginine are much greater than those of histidine. In *Bacillus stearothermophilus* α-amylase, the replacement of a stronger basic residue like arginine with a weaker basic lysine residue disrupted binding of the substrate to the active site (*19*).

The valine moiety of ACV was deemed to be its most flexible portion, as studies have shown that it is possible to generate by replacement with other groups, resulting in unnatural tripeptides that can still be catalysed by IPNS (*2*). Two of these unnatural valine-substituted tripeptides which have been cyclised by IPNS to novel β-lactams are δ-(L-α-aminoadipoyl)-L-cysteinyl-D-α-aminobutyrate (ACAB) (*20*) and δ-(L-α-aminoadipoyl)-L-cysteinyl-D-(*O*-methyl)-D-allothreonine (*21*). Hence, the relaxed substrate specificity of IPNS indicates that substrate-binding ligands may tolerate modifications in ACV, albeit at lower catalytic efficiencies (*2*).

Other studies have also investigated whether a substituted amino acid could replicate the function of the wild-type residue. A study on tomato ACCO on its active site residues showed that changing histidine-177 to glutamate yielded an active enzyme, whereas its replacement with aspartate did not (*22*). Hence, it was suggested that the side-chain length of an amino acid residue might be important for metal binding in ACCO (*22*). However, in the same study on ACCO, when a conservative replacement fo aspartate-179 with glutamate was performed, a low level of <1% of specific activity was observed. Thus, even conservative changes may abolish the specific activity of an enzyme. In another example of a conservative substitution, the replacement of arginine-288 with lysine in a related flavanone 3β-hydroxylase yielded a mutant that was nearly catalytically inactive and retained only 0.092% of the specific activity (*9*). This arginine is involved in the critical binding of the cosubstrate 2-oxoglutarate.

In a recent IPNS review, the active-site ligands of histidine and aspartate were revisited and it was suggested that the two histidines and one aspartate were not interchangeable (*23*). Thus, in addition to a functional role, this HisXAsp(53-57)XHis motif may also have a structural role, although concrete mutational and experimental evidence is yet to be published (*23*). As such, it would be interesting to test this hypothesis using site-directed mutagenesis in either a fungal or bacterial IPNS.

Our results were surprising in showing that the third residue, tyrosine-191, involved in binding the ACV valine carboxylate moiety may be substituted by other amino acids albeit at lowered activity. This suggests that tyrosine-191 is important but not critical like the R-X-S motif, arginine-281 and serine-283, in ACV binding. As a bacterial IPNS crystal structure is still not available, the next rational investigation would be to validate these substrate-binding residues in a bacterial IPNS. Although fungal and bacterial IPNS isozymes share high sequence homology of 55% (*24*), and it is likely that conserved residues have a common

function, only proper experimental evidence would be able to confirm this. As such, we see no lack of impetus in current investigations on IPNS, which is arguably the most studied enzyme (*3*) of this class of non-heme iron oxidases.

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