The isopenicillin N synthase of Cephalosporium acremonium (cIPNS) involves a catalytically important non-heme iron which is coordinated credibly to histidine residues. A comparison of the IPNS genes from various microbial sources indicated that there are seven conserved histidine residues. These were individually replaced by leucine residues through site-directed mutagenesis, and the sites of mutation were confirmed by DNA sequencing. The seven mutant genes were cloned separately into the vector pET24d for expression in Escherichia coli BL21(DE3), and the proteins were expressed as inclusion bodies. The enzymes were purified by ammonium sulfate precipitation and DEAE-Sephadex A-50 ion exchange chromatography, and these were analyzed for enzyme activity. A group of mutant enzymes, H49L, H64L, H116L, H126L, and H137L, were found to be enzymatically active with reduced activities of 16–93.7%, indicating that they are not essential for catalysis. Two of the mutant enzymes, H216L and H272L, were found to have lost their enzymatic activity completely, indicating that both His-216 and His-272 are crucial for catalysis. It is suggested that these histidines are likely to serve as ligands for binding to the non-heme iron in the IPNS active site. Alignment of the amino acid sequence of IPNS to related non-heme Fe²⁺-requiring enzymes indicated that the two essential histidine residues correspond to two invariant residues located in highly homologous regions. The conservation of the two closely located histidine residues indicates the possible conservation of similar iron-binding sites in these enzymes.

In the biosynthesis of the β-lactam antibiotics, penicillin and cephalosporin, one of the key steps is the formation of the β-lactam ring. The enzyme responsible for this reaction is isopenicillin N synthase (IPNS) which catalyzes the oxidative cyclization of δ-L-(α-aminoacyl)-L-cysteinyl-α-valine (ACV) into the first β-lactam intermediate, isopenicillin N. In this reaction, four hydrogen atoms are removed with the subsequent reduction of one molecule of oxygen into water (1). For minimal catalytic activity, the IPNS reaction requires the presence of iron and ascorbate, which indicates that ferrous ions are essential for its enzymatic activity (2). Indeed, results from electron paramagnetic resonance (EPR), Mössbauer, and electronic absorption spectroscopic analyses of the IPNS from Cephalosporium acremonium (cIPNS) have revealed that the active site contains ferrous ions (3). In addition, stoichiometry of one molecule of Fe²⁺ per molecule of the enzyme has been determined for the cIPNS enzyme (3).

Spectroscopic analysis by nuclear magnetic resonance (H NMR) studies have implied that the Fe²⁺ in the native IPNS is six-coordinated with nitrogen- and/or oxygen-containing ligands, of which three could correspond to imidazole moieties (4). The possible involvement of histidine residues as imidazole ligands in the active site of the enzyme was thus suggested. Supporting the role of histidine residues in coordinating Fe²⁺, studies of cIPNS involving multiple scattering analysis of the extended x-ray absorption fine structure data indicated that two or three histidines are ligated to the Fe²⁺ (5). In separate studies, electron spin echo envelope modulation analysis of the Cu²⁺-substituted cIPNS revealed two equatorially coordinated histidines in addition to a water molecule (6).

The IPNS enzyme has been found in a number of prokaryotes and lower eukaryotes known to produce penicillin and cephalosporin. The pcbC gene coding for the IPNS enzyme has been cloned from C. acremonium and Aspergillus nidulans (8), Penicillium chrysogenum (9), Streptomycetes clavuligerus (10), S. lipmanii (11), S. jumonii (11), S. griseus (12), Nocardia lactamurans (13), and Flavobacterium (14). Comparison of the amino acid sequences of these enzymes revealed that they share high homology of 57.1% to 83.1% (15). It has thus been difficult to define amino acids or regions of functional importance. However, since spectroscopic studies have implicated histidine residues as candidates for iron ligands, the conservation of histidine residues could be examined by sequence alignment of all nine known IPNS proteins. In such an analysis, it was observed that, of the ten histidine residues in cIPNS, seven at positions 49, 64, 116, 126, 137, 216, and 272 (with cIPNS sequence as reference) are conserved as illustrated in Fig. 1. We have recently shown that His-272 is essential for the catalytic activity of cIPNS, possibly as a ligand for the non-heme iron (16). In the present study, we used site-directed mutagenesis to assess the importance of each of the other conserved histidine residues for the enzyme activity of recombinant cIPNS expressed in Escherichia coli.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Maintenance of Cultures—The bacterial cultures used were E. coli MV1190, (Δlac-proAB) thi supE Δ(srl-rec)-
Identification of Two His Residues Essential for Catalytic Activity

306:Tn10(tet) F’ [traD36 proAB + lacI lacZAM15] E. coli CJ 236, dut1 ung1 thi-1 relA1 [pc105(Cam’ F’)]; and E. coli BL21(DE3), hsdS5 (λdial857 ind1 sis4 lacUV5-T7). They were maintained on M9 minimal plates or Luria-Bertani (LB) plates (17). LB plates supplemented with 30 μg/mL chloramphenicol were used for the cultivation of E. coli CJ 236. Micrococcus luteus ATCC 381 was maintained and grown on nutrient agar or in nutrient broth.

Site-directed Mutagenesis—Mutagenesis of the cDNS gene was carried out using the Muta-Gene phagemid method. The oligonucleotide-directed mutagenesis was carried out using the T7 polymerase gene that employs the oligonucleotide-directed method. The oligonucleotides used for the mutagenesis were designed as indicated in Table I. The cDNS gene located on a 1690-bp HindIII fragment was cloned into the phagemid vector pTZ19U to create the recombinant vector pmSS891. The mutagenesis was achieved by first annealing the mutagenic oligomer to a uracil-containing pmSS891 template, followed by heteroduplex DNA synthesis (18). The heteroduplex DNA obtained was transformed into competent E. coli MV1190 cells that selected against the uracil-containing template (18). Clones harboring each of the seven histidine site-specific mutated cDNS genes were selected and the single-stranded DNA derived from at least ten clones containing each putative mutant gene were screened by DNA sequencing to confirm the altered base. The single-stranded DNA was prepared by M13K07 superinfection of E. coli MV1190 containing the appropriate phagemid according to Sambrook et al. (17). Sequencing was carried out according to the dideoxy method (19) using the Sequenase 2.0 kit (U. S. Biochemical Corp.).

Construction of Expression Vector—The wild-type cDNS and its various mutant genes were subcloned separately into an expression vector, pET24d (Novagen), which enabled high levels of expression of the cloned cDNS genes in E. coli (20). The pET24d vector employed carries the T7 promoter and translation initiation signals, a unique cloning site, and the T7 transcription terminator, which is inserted downstream of the T7 promoter in the plasmid, pET24d, as indicated in Table I. The cDNS gene located on a 1690-base pair HindII-BamHI fragment was cloned into the phagemid vector pTZ19U to create the recombinant vector pmSS891.

The positions of the base change are underlined.

| Amino acid site | Change in residue | Oligonucleotide primer |
|----------------|------------------|------------------------|
| 49             | His to Leu       | 5'-GACAGCGAGGTTGACACG-3' |
| 64             | His to Leu       | 5'-GCTGATGAGATGTTG-5'   |
| 116            | His to Leu       | 5'-TCCGGGAGGCTCTGGCC-3' |
| 126            | His to Leu       | 5'-GACTCGAGCAATGAG-3'   |
| 137            | His to Leu       | 5'-CCCCGGGAGCTTCCCT-3'  |
| 216            | His to Leu       | 5'-GTCGAGCTACGAG-3'     |
| 272            | His to Leu       | 5'-GCGAGGAGGACTGCGG-3'  |

Relative to ATG start site in cDNS.

HPLC Analysis of IPNS Activity—The methanol-inactivated reaction mixtures were centrifuged at 12,000 rpm for 10 min to remove any precipitated protein before analysis. Separation of the components of the reaction mixture was achieved using the conditions as described by Jensen et al. (23) with the Waters Associate HPLC system which consists of a model 600E solvent delivery system, a model 700 satellite WISP Sample injector, and a model 486 tunable absorbance detector. The mobile phase used for optimal separations was 5% methanol-KH₂PO₄ (0.05 M, adjusted to pH 4.0 with concentrated H₃PO₄) with a flow rate of 0.5 mL/min for 15 min followed by 3 mL/min for the remaining analysis time. UV absorbing materials were detected at 220 nm at a sensitivity of 0.02 absorbance units at full scale. IPNS activity was measured by the reduction of the substrate ACV using DEAE-Sephadex A-50 as the reference standard. After electrophoretic separation of enzymes, all purification procedures were performed at 4 °C. The cell-free extracts containing the recombinant cDNS proteins were concentrated in the 55–85% ammonium sulfate precipitation fractions and dialyzed by passing through a Sephadex G-20 column (Pharmacia, Uppsala, Sweden) (Pharmacia, Uppsala, Sweden). The cDNS proteins were subsequently eluted with a linear concentration gradient of NaCl (0–0.5 M) in Tbuffer (17) TA buffer was then applied onto a 12% SDS-PAGE (21). Fractions containing only the cDNS protein band as seen on denaturing gel were assayed for IPNS activity using the bioassay method. The purified samples were stored at −80 °C with little loss of activity for up to 6 months.

Determination of IPNS Activity by Bioassay Method—The activity of the purified IPNS enzyme was determined by the bioassay method using M. luteus ATCC 318 as the test organism and ACV (a generous gift from Professor S. Wolfe from Simon Fraser University, Canada) as the substrate (22). Reaction mixtures were incubated at 26 °C for 10 min and stopped by the addition of methanol. One unit of activity is the amount of enzyme required to form the equivalent of 1 μmol of isopenicillin N in 1 min.

Purification of Wild-type and Mutant cDNS Proteins—The wild-type and mutant cDNS proteins were purified from the cell-free extracts obtained from 200-mL E. coli cultures using standard procedures involving ammonium sulfate precipitation followed by DEAE-Sephadex A-50 ion exchange chromatography. To minimize heat inactivation of the protein concentrations—protein concentrations were assayed by the Bradford assay (24), using bovine serum albumin (Sigma) as the reference standard. After electrophoretic separation of

![Fig. 1. Alignment of the amino acid sequences of isopenicillin N synthases. The conserved histidine residues are shaded. Amino acid residues are numbered from the first base of the ATG initiation codon.](image)
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RESULTS

Expression of Wild-type and Mutant dPNS Proteins in E. coli—The wild-type C. acremonium IPNS (dPNS) gene was expressed in E. coli BL21(DE3) under the control of the T7 promoter in the vector pET24d. The presence of dPNS in the soluble fraction from the bacterial extracts was detected by SDS-PAGE (Fig. 2A), immunoblot analysis with anti-dPNS serum (Fig. 2B), and by direct measurement of dPNS activity in this fraction after incubation with the substrate ACV (Table II). As shown in Fig. 2A, SDS-PAGE analysis of cell-free extracts derived from E. coli harboring the plasmid with the wild-type dPNS gene revealed an additional protein band of about 38-kDa molecular mass corresponding to the expected dPNS protein (7). The wild-type dPNS enzyme accumulated in the soluble fraction of the cell-free extract was found to reach a concentration of up to 50% of the total soluble proteins. The relative amounts of dPNS produced were measured by scanning densitometry of Coomassie Blue-stained SDS-polyacrylamide gels. Cell lysates from E. coli BL21(DE3) cells transformed with the nonrecombinant vector pET24d alone displayed neither enzymatic activity nor dPNS immunoreactivity (Fig. 2B).

All seven conserved histidine residues in dPNS were modified using site-directed mutagenesis by substituting each of these histidine residues with leucine. After establishing the site of mutation by DNA sequencing (data not shown), the mutated genes were individually cloned into the expression vector pET24d in the same manner as the wild-type dPNS gene and were expressed under similar conditions. SDS-PAGE analysis revealed that the wild-type and the various mutant dPNS proteins exhibited identical mobilities (Fig. 2A). The proteins also possessed similar immunoreactivity toward the antibodies specific to dPNS (Fig. 2B), despite differences in the level of expression.

The enzyme activities of the wild-type dPNS and its mutant enzymes were assayed by HPLC analysis method using cell-free extracts harboring the respective enzymes. As compared to the wild-type enzyme, these mutant enzymes, H49L, H64L, H116L, H126L, and H137L, were found to be enzymatically active but less so than the wild-type enzyme. The relative specific activities of these mutant enzymes ranged from 8.5% to 86.6% as determined by HPLC assay (Table II). The enzyme assays were carried out in duplicate measurements on at least three dilutions of lysates containing the respective mutant proteins and repeated at least four times with substantially the same results in each case. The results obtained indicated that these five histidine residues do not play essential roles in catalysis, although substitution with leucine does influence the level of active enzyme recovered, possibly by affecting enzyme expression, stability, specific activity, or substrate affinity, or a combination of these factors.

No IPNS enzyme activity was detected for the H216L and H272L mutant enzymes in all the determinations by HPLC analysis (Table II). When the same samples were separated on SDS-PAGE and subjected to immunoreactivity to IPNS antisera on Western blots, the amounts of H216L and H272L mutant enzymes detected were found to be comparable with that of the wild-type dPNS (Fig. 2).

Purification and Analysis of Wild-type and Mutant dPNS Enzymes—Further confirmation that two out of the seven mutant dPNS enzymes had loss of activity was obtained after the seven mutant enzymes were purified. The wild-type and mutant dPNS proteins were purified to homogeneity from the cell-free extracts by ammonium sulfate precipitation followed by DEAE-Sephadex A-50 ion exchange chromatography. SDS-PAGE analysis indicated that the dPNS protein was the major component in the 55%-85% ammonium sulfate-saturated fraction (Fig. 3). Purification of each of the mutant dPNS using ion exchange chromatography yielded a single band on SDS-PAGE (Fig. 3). Furthermore, these purified proteins still retained immunoreactivity toward the antibodies specific for dPNS (data not shown). From densitometric scanning of the gel, the purified proteins were estimated to be more than 90% pure.

Enzyme activity determination of the purified wild-type and mutant enzymes by the bioassay method confirmed that alteration of His-49, His-64, His-116, His-126, and His-137 in dPNS resulted in the reduction of enzymatic activities (Table III). The activities of these purified mutant enzymes ranged from 16% to 93.7% relative to that of the wild-type enzyme, while the purified mutant H216L and H272L enzymes did not reveal any catalytic activity. Thus, substitution of His-216 and His-272 by leucine resulted in the production of immunoreactive mutant dPNS that failed to give detectable activity.
Identification of Two His Residues Essential for Catalytic Activity

Enzyme activity was measured using HPLC assay and is expressed as the amount of ACV monomer consumed after a 10-min reaction/mg of protein of the cell-free extracts. The data represented here are from one experiment in which measurements were done in duplicates using two or three dilutions of each cell-free extract.

| Enzyme type | Amount of proteins used in assay (µg) | Amount of ACV monomer consumed (µmol) | Specific activity (µmol ACV/mg/min) | Relative specific activity based on total proteins (%) | Relative percent of IPNS expressed (%) | Relative specific activity based on IPNS protein (%) |
|-------------|-------------------------------------|--------------------------------------|-------------------------------------|------------------------------------------------------|--------------------------------------|------------------------------------------------------|
| Wild-type   | 3.19                                | 0.121                                | 3.78                                | 100                                                  | 100                                  | 100                                                  |
| H49L        | 5.64                                | 0.07                                 | 1.25                                | 3.3                                                  | 38.7                                 | 8.5                                                  |
| H64L        | 2.54                                | 0.074                                | 2.81                                | 77.1                                                 | 89.0                                 | 86.6                                                 |
| H116L       | 2.15                                | 0.0091                               | 0.42                                | 11.1                                                 | 26.1                                 | 42.5                                                 |
| H126L       | 2.02                                | 0.0109                               | 0.54                                | 14.3                                                 | 28.3                                 | 50.5                                                 |
| H137L       | 4.36                                | 0.094                                | 2.16                                | 57.0                                                 | 76.4                                 | 74.6                                                 |
| H216L       | 13.9                                | 0.0                              | 0                                    | 0                                                    | 100                                  | 0                                                    |
| H272L       | 10.2                                | 0                                   | 0                                    | 0                                                    | 100                                  | 0                                                    |

a Relative specific activity expressed as specific activity relative to that of the wild-type.
b Percent amount of IPNS protein expressed relative to that of the wild-type protein as measured by scanning densitometry (raw data not shown).
c Relative specific activity of IPNS was calculated as a percentage of the relative activity based on total proteins to the relative percent of IPNS expressed (arb).

Fig. 3. SDS-PAGE analysis of the purified wild-type and mutant cIPNS enzymes. The proteins were purified through ammonium sulfate precipitation and DEAE-Sephadex A-50 ion exchange chromatography. The molecular mass markers (in kDa) are indicated on the left. Lane 1 shows the cell-free extract from E. coli expressing the wild-type dPNS, and lane 2 shows the 55–85% ammonium sulfate-saturated fraction of the cell-free extract. The following lanes were each loaded with the purified proteins as indicated: in lane 3, wild-type dPNS; lane 4, H49L-dPNS; lane 5, H64L-dPNS; lane 6, H116L-dPNS; lane 7, H126L-dPNS; lane 8, H137L-dPNS; lane 9, H216L-dPNS; lane 10, H272L-dPNS. The gel was stained with Coomassie Brilliant Blue dye.

Discussion

Since the IPNS gene was first cloned from C. acremonium (7), there has been much interest in determining functionally important amino acids involved in the catalytic function of IPNS. Thus, much information has been accrued from studies of the primary structure of IPNS (7–14), spectroscopic analysis of the enzyme (3–6, 26–28), affinity labeling of the substrate binding sites (29), chemical modification of reactive groups (2), and site-directed mutagenesis studies (16, 30). Based on chemical modification studies (2), cysteine residues were thought to play an important role in the IPNS catalytic center. However, site-directed mutagenesis of two cysteine residues, Cys-106 and Cys-255 of cIPNS, followed by enzyme activity studies revealed that they were not essential for IPNS activity (30). This has led us to investigate the functional role of histidine residues in dPNS through site-directed mutagenesis of conserved histidine sites since spectroscopic studies have implicated the involvement of histidines as iron ligands (3–6, 26–29).

Thus, in our previous analysis, a catalytically important residue His-272 of dPNS was proposed as a possible ligand for iron binding (16). In this study, evaluation of the activity of the same H272L mutant enzyme by HPLC determination and analysis of the purified enzyme confirmed further that this mutant enzyme lacks any detectable activity, thereby substantiating the importance of His-272 for IPNS catalysis.

The search for conserved histidine residues that might be functional in enzyme catalysis has been investigated by amino acid sequence comparisons of a number of related non-heme Fe²⁺-requiring enzymes including the IPNS enzymes (Fig. 4). These studies indicated that His-49 (31, 33), His-216 (32, 33), and His-272 (31–33) of dPNS are conserved in regions of relatively high sequence homology. Among the enzymes compared were 2-oxoglutarate-dependent dioxygenases (prolyl and lysyl hydroxylase) from animal primary metabolism, 2-oxoglutarate-dependent hydroxylases (hyocyanamine 6β-hydroxylase, flavanone 3β-hydroxylase, deacetoxycephalosporin C synthase, and deacetylcephalosporin C synthase) from plant, fungal, and bacterial secondary metabolism, and enzymes involved in ethylene formation and anthocyanidin biosynthesis in plants. In a number of these enzymes, e.g. prolyl hydroxylases and flavanone hydroxylases, the importance of histidine residues to their catalytic mechanism has been demonstrated through chemical modification studies (32, 33). In addition, it was demonstrated recently that site-directed mutagenesis of human prolyl 4-hydroxylase (34) identified three histidine residues to be critical for activity, possibly as ligands for iron binding, and two of these correspond to the two essential histidine residues that have been experimentally shown to be important for the catalysis of dPNS. One similar feature of these enzymes including IPNS is that they require ferrous iron and oxygen for activity. In view of the common requirement for ferrous iron to invoke activity in all the enzymes studied, albeit from uncommon sources, it appears that the homologous sequences in the vicinity of certain histidine residues of these enzymes might represent part of the iron-binding site. The conservation of the histidine residues provides strong support for the possible conservation of similar iron-binding sites in these enzymes.

A recent analysis involved the comparison of the sequences of penicillin and cephalosporin biosynthetic enzymes, viz. nine IPNS enzymes from various microbial sources, deacetoxycephalosporin C synthase and deacetylcephalosporin C synthase from S. clavuligerus, and the C. acremonium deacetoxycephalosporin C synthase (35). From the alignment of the primary protein structure of these enzymes and a comparison of their putative secondary structures, two histidine residues equivalent to His-216 and His-272 of dPNS were found to be aligned consistently in the 12 proteins compared. These two histidine residues were thereupon predicted to have the highest probability of being iron-binding ligands.

Our experimental investigations are in support of this predictive analysis and have also identified His-216 and His-272 in dPNS as being necessary for the catalytic activity of dPNS.
Identification of Two His Residues Essential for Catalytic Activity

The IPNS activity was determined by the bioassay method. The activity assays were performed using two different preparations of purified samples, with duplicate measurements.

| Enzyme type | Protein concentration | IPNS activitya | Speciﬁcity | Relative percent speciﬁcityb |
|-------------|----------------------|----------------|-------------|-----------------------------|
| Wild-type   | 20.5                 | 0.279          | 13.6        | 100                         |
| H49L        | 15.5                 | 0.035          | 2.24        | 16                          |
| H46L        | 18.5                 | 0.233          | 12.7        | 93.7                        |
| H316L       | 24.5                 | 0.285          | 11.6        | 85.4                        |
| H126L       | 18.5                 | 0.191          | 10.3        | 75.7                        |
| H137L       | 21.0                 | 0.235          | 11.2        | 82.1                        |
| H216L       | 43.5                 | 0.0            | 0           | 0                           |
| H272L       | 41.0                 | 0.0            | 0           | 0                           |

a One unit of activity is the amount of IPNS required to form the equivalent of 1 \( \mu \text{mol} \) of isopenicillin N/ml/min at 26°C.

b Relative speciﬁcity expressed as percentage of speciﬁc activity relative to that of the wild-type enzyme.

![Comparison of amino acid sequences of IPNS enzymes and related enzymes showing homologous regions with the conserved histidine residue (*)](Image)
liver phenylalanine hydroxylase which requires a non-heme Fe$^{2+}$ for the conversion of phenylalanine to tyrosine, two histidine residues have also been proposed to bind the iron (38). Further studies on the iron content or iron affinity in the site-specific mutant proteins is warranted for elucidating a clearer role for the paired histidines in IPNS.

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