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Analysis of the role of cysteine residues in isopenicillin N synthetase activity by site-directed mutagenesis

(*Cephalosporium acremonium*/β-lactam biosynthesis/iron binding)

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ABSTRACT **The predicted amino acid sequences of isopenicillin N synthetase from both *Cephalosporium acremonium* and *Penicillium chrysogenum* have two cysteine residues in analogous positions (Cys-106 and Cys-255 in the *C. acremonium* numbering). To examine the role of these cysteine residues in the activity of the *C. acremonium* enzyme, we used site-directed *in vitro* mutagenesis to change these cysteine residues to serine residues. Mutation of Cys-255 reduces specific activity ≈50%, whereas mutation of Cys-106 or mutation of both Cys-106 and Cys-255 reduces specific activity about 97%. This suggests that the cysteines are important but not essential for IPNS activity. Alkylation of IPNS also almost completely inactivated the enzyme, but residual activity could have been due to incomplete alkylation. Atomic substitution via genetic manipulation in this case is a more accurate means of assessing the role of sulfhydryl moieties in enzyme activity.**

The biochemical literature is replete with studies claiming an essential role for sulfhydryl groups in the activity of particular enzymes. These conclusions are usually based on studies utilizing agents, such as *p*-chloromercuribenzoate or iodoacetate, that alkylate the sulfhydryl moiety on cysteine residues. This method, while useful, suffers from the inability to distinguish between the effect of blocking the reactivity of the sulfhydryl by alkylation and the steric effect of the attached alkyl moiety. Also, residual activity after alkylation could be due to incomplete alkylation or intermediate effects of alkylation on activity. Use of recombinant techniques to readily replace cysteine residues with serine residues avoids most if not all of the steric effects. Thus, when activity is observed with the serine-analog enzymes, hypothetical mechanisms including sulfur-sulfur bonds within the enzyme or between enzyme and substrate can be eliminated. The utility of this approach is illustrated by the preparation of three serine analogs of isopenicillin N synthetase (IPNS).

IPNS is a key enzyme in the biosynthesis of penicillins and cephalosporins. These β-lactam antibiotics are clinically important compounds because they effectively kill bacteria by inhibiting cell-wall synthesis and have low toxicity in mammals. IPNS catalyzes the condensation of the tripeptide δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (δ-Aad-Cys-D-Val) to isopenicillin N. During the course of this unusual reaction, shown in Fig. 1, four hydrogen atoms are removed from the substrate, one molecule of oxygen is consumed, and a two-ring system is formed. Previous characterization of IPNS has shown that iron is a required cofactor (1). Certain reagents that covalently modify cysteine residues, including 2-pyridyl disulfide and *N*-ethylmaleimide but not Ellman's reagent, will almost completely inactivate the enzyme, and the inactivation by 2-pyridyl disulfide can be reversed by

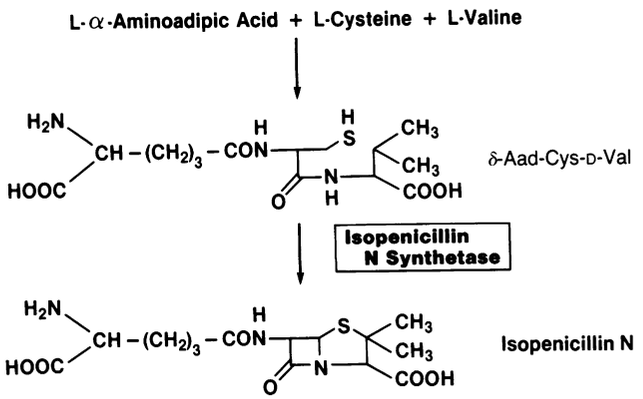

FIG. 1. Reaction catalyzed by IPNS.

dithiothreitol (D. Perry and E. P. Abraham, personal communication). These observations suggest that the sulfhydryl moiety on a cysteine side chain may be involved in catalysis, iron binding, or both. To further elucidate the role of sulfhydryls in IPNS activity, we have constructed three serine analogs of the *Cephalosporium acremonium* IPNS and measured their catalytic activities.

We have previously reported the cloning and sequencing of the *C. acremonium* IPNS gene (2). The amino acid sequence predicted from the DNA sequence contains two cysteine residues, Cys-106 and Cys-255. Codons for these cysteine residues are also found in analogous positions in the closely related IPNS gene from *Penicillium chrysogenum* (3). To evaluate the importance of these cysteine residues, we have used site-directed *in vitro* mutagenesis to change these cysteine residues to serine residues. Serine was chosen because the shape of the molecule is very similar to cysteine, but instead of a reactive sulfur atom serine contains an oxygen atom. Because IPNS activity of the mutant enzymes is significantly reduced but not completely lost, we have concluded that the cysteine residues are important but not necessary for IPNS activity.

MATERIALS AND METHODS

Mutagenesis. The *C. acremonium* IPNS gene was cloned into bacteriophage M13mp19 (4) on a 2.7-kilobase (kb) *Bam*HI fragment to create mIT110 (Fig. 2). This *Bam*HI fragment was isolated from pIT335, which has been described (2).

Oligonucleotides for mutagenesis and sequencing were synthesized using an Applied Biosystems DNA synthesizer (model 380A) according to the manufacturer's recommended

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Abbreviations: IPNS, isopenicillin N synthetase; δ-Aad-Cys-D-Val, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine.

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protocols. Oligomers were purified by 20% polyacrylamide/7 M urea gel electrophoresis. Mutagenesis was carried out (with minor modifications) according to the method described by Adelman *et al.* (5), which employs nuclease S1 to reduce the background of wild-type sequences. The mutagenic linkers incorporated a cytosine residue instead of a guanidine residue at the second position of the cysteine codon, as shown in Fig. 3. Mutants were detected by determining the pattern of cytosine residues in random isolates by dideoxy sequencing (15) and looking for an extra cytosine residue relative to wild type. The DNA sequence around the site of mutagenesis was then determined for all four bases for some of the promising isolates, using the chemical cleavage method of Maxam and Gilbert (6).

Expression of IPNS Genes in *Escherichia coli*. The mutant IPNS genes were subcloned into an expression vector so the protein could be expressed in *E. coli*. The cloning scheme is shown in Fig. 2. Mutant genes were isolated on an *Nco* I–*Bam*HI fragment [the translation initiation site of the IPNS gene is nested within the *Nco* I site; the *Bam*HI site is about

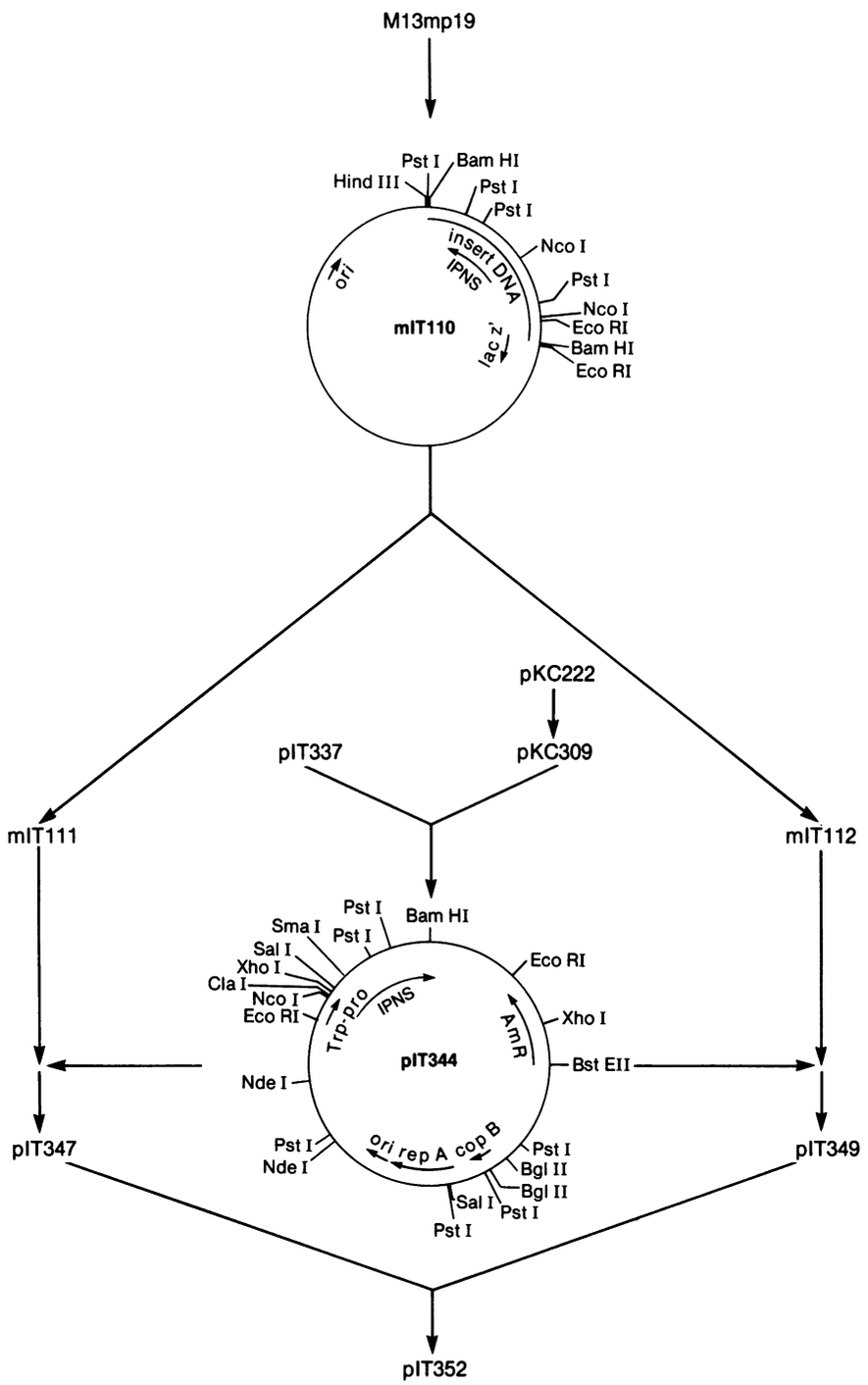

FIG. 2. Plasmid and phage constructs used for mutagenesis and expression in *E. coli*. The *C. acremonium* IPNS gene was subcloned into M13mp19 to create mIT110, and the cysteine codons in mIT110 were mutagenized to serine codons (as detailed in Fig. 3 and *Materials and Methods*). mIT111 contains the derivative altered at Cys-106, and mIT112 contains the mutation at Cys-255. The IPNS genes were expressed under transcriptional control of the *E. coli* tryptophan promoter (Trp-pro) on a plasmid containing a temperature-sensitive copy-control system. The wild-type gene is contained on pIT344, the mutation at Cys-106 is contained on pIT347, the mutation at Cys-255 is contained on pIT349, and a gene containing a mutation at both Cys-106 and Cys-255 is contained on pIT352. AmR, apramycin-resistance gene.

400 base pairs (bp) downstream of the translation termination site] and cloned into pIT344. Plasmid pIT344 is a derivative of pIT337 (2) constructed by replacing the kanamycin-resistance gene in pIT337 with an apramycin-resistance gene as follows. The apramycin-resistance gene was contained on a 1.6-kb *Xmn* I fragment from pKC309 (R. N. Rao, personal communication) and was originally obtained from pKC222 (7). pIT337 was digested with *Kpn* I and *Bst*EII, treated with phage T4 DNA polymerase to produce blunt ends (8), and ligated with the 1.6-kb *Xmn* I fragment. The resulting plasmid, pIT344, contains the wild-type IPNS gene fused to the *E. coli* tryptophan promoter and a temperature-sensitive copy-control gene ("runaway replicon").

Determination of IPNS Activity. IPNS activity was determined as described previously (3). The amount of IPNS protein used in the assay was calculated by determining the fraction of IPNS in the partially purified preparation and multiplying this fraction times the total amount of protein used in the assay. The percent IPNS in the sample was determined by $NaDodSO_4$/PAGE of the partially purified preparation, followed by scanning densitometry the Coomassie blue R-250-stained gel and integration of the peak heights. Total protein concentrations were determined by a Coomassie blue G-250 binding assay (9). Immunological detection of IPNS protein was done as described (3).

RESULTS

Construction of Mutant Alleles. Site-directed mutagenesis was used to change cysteine codons to serine codons in the *C. acremonium* IPNS gene. The IPNS gene was cloned into M13mp19 to create mIT110 (Fig. 2), and the single-stranded form of mIT110 was used for mutagenesis. Mutagenic 36-base oligonucleotides containing a single mismatch with the wild-type sequence were employed as described in *Materials and Methods*. The oligonucleotide sequence and the predicted amino acid sequence changes are shown in Fig. 3. Derivatives of mIT110 containing the desired mutations were identified by sequencing. The mutation at Cys-255 could also be identified by *Hpa* II restriction endonuclease digestion, because a new *Hpa* II site is created by the mutation. Twenty-four random phage plaques were isolated from each of the two pools after mutagenesis and the pattern of cytosine residues near the cysteine codons was determined by dideoxy sequencing. Seventeen out of 24 (71%) had the desired mutation at Cys-106, and 8 out of 24 (33%) had the desired mutation at Cys-255. mIT111 was chosen as a representative clone containing the mutation at Cys-106, and the mutation at Cys-255 is contained in mIT112. The DNA sequence of the mutant alleles near the site of the mutation was determined by Maxam–Gilbert sequencing (6) (Fig. 4).

Construction of Expression Vectors. The mutant IPNS genes were cloned from the M13 clones to a plasmid vector for expression in *E. coli* (see *Materials and Methods* and Fig. 2). A mutant allele containing mutations in both cysteine codons was created by *in vitro* splicing of fragments from pIT347 and pIT349 (Fig. 2). An *Avr* II site, unique in pIT344, is found between the cysteine codons in the IPNS gene. First, a 980-bp *Avr* II–*Bam*HI fragment containing the carboxyl-terminal portion of the IPNS gene (including the Cys-255 → Ser-255 mutation) was purified from pIT349. Next, an 8340-bp *Avr* II–*Bam*HI fragment containing the amino-terminal portion of the IPNS gene (including the Cys-106 → Ser-106 mutation) and all plasmid functions was purified from pIT347. After ligation of the two fragments, a derivative was obtained that contained an extra *Hpa* II site relative to pIT347, which is indicative of the mutation at Cys-255 (see Fig. 3). This plasmid, called pIT352, contains mutations at both cysteine codons of the IPNS gene.

Expression of Mutant Alleles. The IPNS proteins were expressed in *E. coli* by growing the cells at 25°C to an optical density of about 0.1 (at 600 nm) and then raising the temperature to 37°C for 6 hr, as described (2). IPNS protein accumulated to 10–20% of total cell protein under these conditions, as measured by quantitative scanning of Coomassie blue-stained polyacrylamide gels of total cell proteins. The IPNS protein accounted for 20–50% of the protein in an 85% ammonium sulfate cut of total soluble protein. The identity of the IPNS band in the polyacrylamide gels was proven by immunoblotting (Fig. 5). The relative amount of IPNS protein in extracts was the same when determined by immunological analysis or Coomassie blue staining (data not shown).

Activity of Mutant Proteins. The activity of the wild-type and mutant IPNS proteins is summarized in Table 1. Mutant IPNS that has two serine residues in place of the two cysteine residues of wild-type IPNS can catalyze the condensation of δ-Aad-Cys-D-Val to isopenicillin N; however, its specific activity is reduced by a factor of ≈30 relative to wild type. Almost all of the reduction in the specific activity seems to be due to the loss of Cys-106, since changing Cys-106 to serine decreased the specific activity by a factor of 20. Changing

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amino acid#        100                        106            110
amino acid     LYS LYS ALA VAL GLU SER PHE CYS TYR LEU ASN PRO SER PHE SER
pIT344      ...AAG AAG GCG GTC GAA TCG TTC TGC TAC CTG AAC CCC TCC TTC AGC
mutagenic           AG GCG GTC GAA TCG TTC TCC TAC CTG AAC CCC TCC T
oligo
amino acid                           .....SER.....
change

amino acid#             250            255                 260
amino acid     ASP THR GLY PHE LEU ILE ASN CYS GLY SER TYR MET ALA HIS ILE
pIT344      ...GAC ACG GGC TTC CTC ATC AAC TGC GGC AGC TAC ATG GCC CAT ATC
mutagenic           CGGGC TTC CTC ATC AAC TCC GGC AGC TAC ATG GCC
oligo
amino acid change                    .....SER.....
```

FIG. 3. Details of the mutagenesis of the IPNS gene. The two cysteine codons were mutated and characterized separately as described in *Materials and Methods*. The mutant alleles were then spliced to form the double mutant (see Fig. 2). The sequence listed for pIT344 is that of the wild-type IPNS gene.

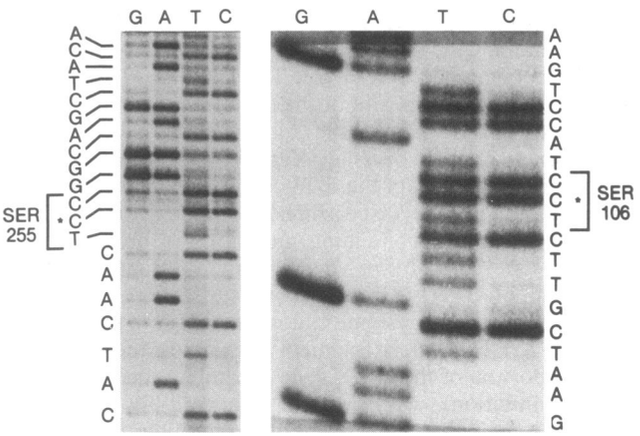

FIG. 4. Sequence analysis of the mutant alleles of the IPNS gene. The sequence of the mutant alleles was determined around the site of the mutation by the chemical cleavage method of Maxam and Gilbert (6). The mutated bases are indicated by stars; the sequence of the wild-type gene is given in Fig. 3.

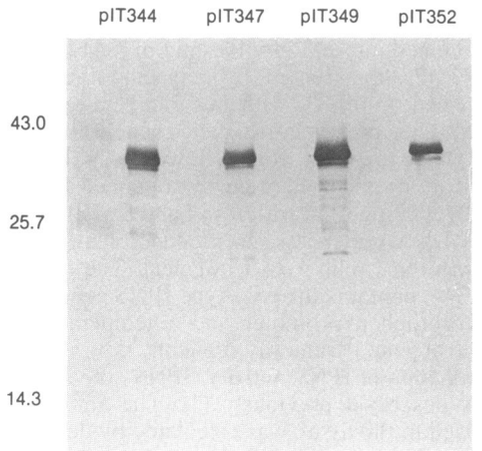

FIG. 5. Proof of expression of IPNS alleles in *E. coli* by immunological analysis. Partially purified protein extracts from *E. coli* cells containing the indicated plasmids were prepared, fractionated by $NaDodSO_4$/PAGE, and analyzed with polyclonal antibodies after electroblotting. Equal amounts of *E. coli* extract protein were loaded in each lane so that the amounts of IPNS were comparable. Positions of molecular weight standards ($M_r \times 10^{-3}$) run in parallel are given at left.

Cys-255 to serine in the wild-type protein caused only a 50% decrease in specific activity, and changing Cys-255 to serine in the Ser-106 mutant caused a relatively small drop in specific activity relative to the specific activity of the singly mutated protein. Changing Cys-106 to a serine residue was associated with a 5-fold increase in the K_m, whereas changing Cys-255 to a serine residue had virtually no effect on the K_m.

DISCUSSION

The IPNS protein catalyzes the condensation of the two-ring system of isopenicillin N. Iron and molecular oxygen are required for activity, and reagents that covalently attach to sulfhydryls almost completely inactivate the protein. Since the IPNS reaction involves formation of a carbon-sulfur bond in the five-membered thiazolidine ring, several hypothetical roles for the sulfhydryls in IPNS activity can be advanced. Four possibilities are shown schematically in Fig. 6: (*a*) the sulfhydryl(s) may simply play a structural role, stabilizing the proper conformation of the protein; (*b*) the sulfhydryl(s) may carry out a catalytic role, directly involved in electronic interaction with the substrate in the active site; (*c*) the sulfhydryl(s) may be indirectly involved in catalysis, perhaps by binding and/or stabilizing the substrate or a cofactor such as iron or molecular oxygen; or (*d*) the role of the sulfhydryls may be some combination of the above.

To distinguish among these possibilities, we changed the cysteine residues in IPNS to serine residues, either singly or in combination. Serine was chosen because it has almost the same spatial configuration as cysteine, thereby minimizing any conformational perturbation that might be introduced by a differently shaped side chain.

We found that IPNS is able to catalyze condensation of δ-Aad-Cys-D-Val to isopenicillin N even when both sulfur atoms have been replaced by oxygen atoms, although the specific activity is reduced by a factor of ≈30. The substantial loss of activity argues against hypothesis *a*, since substitution of a serine for a cysteine should not significantly affect the conformation of a protein, especially the conformation of an intracellular protein, which is unlikely to form intramolecular disulfides because of the reducing environment inside the cell. The *in vitro* IPNS reaction is also carried out under conditions that reduce disulfide bonds. In addition, our results suggest little functional interaction between the two sulfhydryls in IPNS, since alteration of Cys-255 has little effect on the protein, with or without the additional alteration of Cys-106. The fact that IPNS is active even in the absence of both sulfur atoms argues against hypothesis *b*, since the chemical reactivity of the oxygen atom in the hydroxyl group of serine is substantially different from the reactivity of the sulfur atom in the sulfhydryl of cysteine. It is unlikely that oxygen could substitute for sulfur if, for example, part of the catalytic involvement of the sulfur atom was in formation of an intermediate disulfide bond with the substrate, as shown schematically in Fig. 6*b*.

The most likely role for the sulfhydryls in IPNS is therefore the intermediate role in catalysis proposed in Fig. 6*c*. Perhaps the sulfur atom from Cys-106 is involved in binding, stabilizing, or activating a cofactor. The absolute requirement for iron for activity suggests that the sulfhydryl might be involved in binding iron to the protein. For example, the

Table 1. Specific activities of wild-type and mutant IPNS proteins

Plasmid	Genotype	IPNS, μg per well	Zone size, mm	Isopenicillin equivalents, nmol	Specific activity, units/mg of IPNS	K_m, mM
pIT344	Wild type	2.4	27	14.20	0.59	0.15
pIT347	Ser-106	10.0	16	2.71	0.03	0.70
pIT349	Ser-255	2.0	23	5.70	0.29	0.15
pIT352	Ser-106, Ser-255	12.5	13	2.10	0.02	0.77

Activity was measured as described in ref. 3. One unit of IPNS is the amount necessary to convert 1 μmol of δ-Aad-Cys-D-Val to isopenicillin N in 1 min at 25°C. Enzymatic reactions were 10-min assays; the specific activity values take this into account.

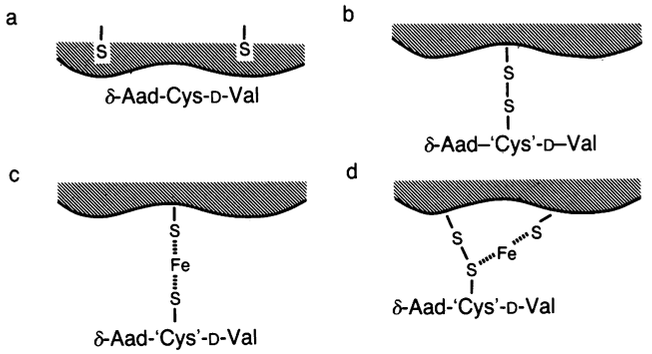

FIG. 6. Hypothetical roles for sulfhydryl groups in IPNS. Hatched region represents the enzyme. In *b–d*, the sulfur of the cysteine residue in the substrate δ-Aad-Cys-D-Val is shown explicitly; hence the three-letter symbol for cysteine is shown in quotes.

sulfhydryls of cysteines are believed to be involved in binding iron-sulfur clusters to the apoprotein in enzymes such as beef heart aconitase (10) and ferredoxin (11). This would explain why covalent modification of the sulfhydryl groups dramatically reduced activity of the protein, since the addition of an alkyl group might sterically exclude the iron from the active site, whereas substitution of an oxygen for the sulfur would still allow the iron to reach the active site. Another possibility is that the sulfhydryl is involved in substrate binding but not directly involved in catalysis. This possibility can be tested by measuring the K_m values for binding of substrate in the wild-type and mutant proteins; if Cys-106 is involved in substrate binding, it is likely that the K_m for substrate binding to the Ser-106 mutant should be increased relative to the K_m for the wild-type protein. In fact, we found that the K_m for proteins with the Ser-106 mutation is about 5-fold higher than for proteins with a cysteine at position 106. Thus it appears likely that the cysteine residue at 106 is involved in substrate binding, either directly or indirectly. The iron-binding and substrate-binding hypotheses are not mutually exclusive; perhaps part of the binding energy for substrate to enzyme binding is mediated through an iron–substrate interaction, and Cys–106 is involved in binding of the iron to the protein. Proof of the involvement of sulfhydryl groups in iron binding could be obtained from x-ray spectroscopic techniques (e.g., ref. 12) or ultimately from x-ray crystallographic analysis.

Inactivation studies using sulfhydryl-reactive alkylating agents would have led to the conclusion that the *C. acremonium* IPNS contains an essential cysteine residue. In fact, IPNS activity is easily demonstrable from the doubly mutated gene on pIT352, which does not encode any cysteine residues. These observations suggest that conclusions concerning the relative importance of cysteine residues based on alkylation experiments (e.g., ref. 13) must be drawn cautiously. An example of clear demonstration of an essential sulfhydryl residue is the genetic replacement of cysteine by glycine in the *Serratia marcescens* anthranilate synthase (14). Replacement of a single cysteine by glycine caused a total loss (less than 0.03% of wild-type activity) of glutamine-dependent anthranilate synthase activity. By contrast, Cys-106 in the IPNS enzyme is important but not essential.

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