

# Ferrous Active Site of Isopenicillin N Synthase: Genetic and Sequence Analysis of the Endogenous Ligands<sup>†</sup>

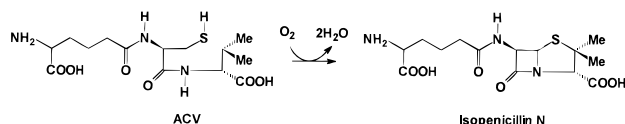
Ilya Borovok,<sup>‡</sup> Orna Landman,<sup>‡</sup> Rachel Kreisberg-Zakarin, Yair Aharonowitz, and Gerald Cohen\*

Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Ramat Aviv, 69978, Israel

Received July 6, 1995; Revised Manuscript Received November 7, 1995<sup>⊗</sup>

**ABSTRACT:** Isopenicillin N synthase (IPNS) from *Streptomyces jumonjinensis* ( $M_r$  37 902) is a non-heme ferrous iron-containing enzyme that catalyzes the oxidative cyclization of the tripeptide  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-valine (ACV) to form isopenicillin N. Spectroscopic studies [reviewed in Cooper, R. D. (1993) *Biomed. Chem. I*, 1–17] have led to a model for the coordination environment of the iron atom possessing three histidine and one aspartic acid endogenous ligands and a solvent molecule. A refinement of that model proposes that formation of the Fe(II) IPNS–ACV complex occurs with displacement of the H<sub>2</sub>O from the metal center and that one of the histidines is subsequently replaced by a solvent molecule on binding of dioxygen. Here we report genetic studies to determine the nature and location of the endogenous ligands in the *S. jumonjinensis* IPNS primary amino acid sequence that constitute the ferrous active site. Site-directed mutagenesis was used to exchange each of the seven histidines and the five aspartic acids that are conserved in bacterial and fungal IPNS proteins. Biochemical analysis of the alanine-substituted mutant proteins shows that two histidines, His212 and His268, and one aspartic acid, Asp214, are essential for enzyme activity. The other mutant enzymes have specific activities 5–68% that of wild type. Sequence analysis of 10 IPNS and 42 other non-heme ferrous iron-dependent dioxygenases reveal the presence of a common motif, **HisXAsp(53–57)XHis**, which in IPNS contains the identical two histidines and one aspartic acid essential for function. Accordingly, we have assigned residues His212, His268, and Asp214 as three of the four endogenous ligands postulated to form the IPNS ferrous active site. Compelling support for these conclusions comes from the recent crystal structure determination of the manganese form of a fungal IPNS [Roach et al. (1995) *Nature* 375, 700–704].

A key step in the biosynthesis of penicillin and cephalosporin antibiotics is the oxidative cyclization of  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-valine (ACV)<sup>1</sup> by isopenicillin N synthase (IPNS) to form the  $\beta$ -lactam and thiazolidine rings of isopenicillin N (Baldwin, 1989). IPNS belongs to the class of non-heme Fe(II)-containing enzymes that catalyze a wide range of reactions including aliphatic hydroxylations, desaturations, and cyclizations of rings containing heteroatoms through activation of dioxygen (Ingraham & Meyer, 1985; Que, 1989). However, unlike most of these Fe(II)-dependent dioxygenases which incorporate oxygen atoms into their substrates, IPNS completely reduces dioxygen to 2 equiv of H<sub>2</sub>O in the cyclization of ACV (White et al., 1982; Baldwin & Abraham, 1988).



The mechanism of IPNS catalysis has been extensively examined by studies of the kinetics and stereochemistry and by the use of a large number of substrate analogs (Baldwin & Abraham, 1988; Robinson, 1988; Baldwin & Bradley,

1990). More recently, spectroscopic techniques have been employed to define the coordination environment of the ferrous active site. The combined results of EPR, Mossbauer, NMR, and electronic spectroscopy of Fe(II) IPNS and its ACV complex, and the corresponding Co(II) and Cu(II) derivatives, support a metal center containing three endogenous histidine and one aspartic acid ligands and exogenous sites for ACV, dioxygen, and solvent (Chen et al., 1989; Ming et al., 1990; Jiang et al., 1991; Ming et al., 1991). A refinement of this model proposes that the axial histidine ligand in the Fe(II) IPNS–ACV complex is displaced on oxygen binding (Ming et al., 1991), leaving the two remaining histidines in the equatorial plane of the metal center. X-ray absorption studies of Fe(II) IPNS and its substrate complex confirm the main details of the model (Scott et al., 1992; Randall et al., 1993) and verify that ACV is bound to the iron atom in the active site through the cysteinyl sulfur (Orville et al., 1992).

In this paper we describe a site-directed mutagenesis study to identify the specific histidine and aspartic acid residues in the IPNS primary amino acid sequence that constitute the endogenous ligands of the ferrous active site. Multiple sequence alignment of 10 bacterial and fungal IPNS proteins shows that seven histidines and five aspartic acids are

<sup>†</sup> This research was supported by Grant 93-00097/1 from the U.S.–Israel Binational Science Foundation, Jerusalem, Israel, and in part by a grant to I.B. from the Minerva Foundation.

\* Corresponding author.

<sup>‡</sup> I.B. and O.L. should be considered equal first authors.

<sup>⊗</sup> Abstract published in *Advance ACS Abstracts*, January 15, 1996.

<sup>1</sup> Abbreviations: ACV,  $\delta$ -(L- $\alpha$ -aminoadipoyl)-L-cysteinyl-D-valine; IPNS, isopenicillin N synthase; ACCO, 1-aminocyclopropane-1-carboxylic acid oxidase; SDS, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

		14			48	
ipns_emeni	MGVSVK..AN	VPKIDVSPFLF	GDDQAAKMRV	AQQIDAASRD	TGFFYAVNHG	INVQRLSQKT
ipns_pench	MASTPK..AN	VPKIDVSPFLF	GDNMEEEKMKV	ARAIIDAASRD	TGFFYAVNHG	VDVKRLSNKT
ipns_cepac	MGSPVPVPAN	VPRIDVSPFLF	GDDKEKKLEV	ARAIIDAASRD	TGFFYAVNHG	VDLPWLSRET
ipns_strju	.MPILMPSAE	VPTIDISPLS	GDDAKAKQRV	AQEINKAARG	SGFFYASNHG	VDVQLLQDVV
ipns_strcl	.MPVLMPSAH	VPTIDISPLF	GTDAAAKKRV	AEEIHGACRG	SGFFYATNHG	VDVQQLQDVV
ipns_nocla	...MKMPSAE	VPTIDVSPFLF	GDDAQEKVRV	GQEINKACRG	SGFFYAANHG	VDVQRLQDVV
ipns_strgr	.MPIPMLPAH	VPTIDISPLS	GGDADDKKRV	AQEINKACRE	SGFFYASHHG	IDVQLLKDVV
ipns_strlp	.MPVLMPSAD	VPTIDISPLF	GTDPDAKAHV	ARQINEACRG	SGFFYASHHG	IDVRRLLQDVV
ipns_flass	...MNRHAD	VPVIDISGLS	GNDMDVKKDI	AARIDRACRG	SGFFYAANHG	VDLALQKFT
		63			113 114	
ipns_emeni	KEF <b>H</b> MSITPE	EKWDLAIRAY	NKEHQDQVRA	GYLSIPGKK	AVWSFCYLNP	NFTPD <b>H</b> PRIQ
ipns_pench	REF <b>H</b> FSITDE	EKWDLAIRAY	NKEHQDQIRA	GYLSIPEKK	AVESFCYLNP	NFKPD <b>H</b> PLIQ
ipns_cepac	NKF <b>H</b> MSITDE	EKWDLAIRAY	NKEHESQIRA	GYLPIPGKK	AVESFCYLNP	SFSPD <b>H</b> PRIK
ipns_strju	NEF <b>H</b> RNMSDQ	EKHDLAINAY	NKDN.PHVRN	GYKAIKGKK	AVESFCYLNP	SFSDD <b>H</b> PMIK
ipns_strcl	NEF <b>H</b> GAMTDQ	EKHDLAIHAY	NPDN.PHVRN	GYKAVPGRK	AVESFCYLNP	DFGED <b>H</b> PMIA
ipns_nocla	NEF <b>H</b> RTMSPQ	EKYDLAIHAY	NKNN.SHVRN	GYMATEGKK	AVESFCYLNP	SFSE <b>DH</b> PEIK
ipns_strgr	NEF <b>H</b> RTMTDE	EKYDLAINAY	NKNN.PRTRN	GYMAVKGKK	AVESWCYLNP	SFSE <b>DH</b> PQIR
ipns_strlp	NEF <b>H</b> RTMTDQ	EKHDLAIHAY	NENN.SHVRN	GYMARPGRK	TVESWCYLNP	SFGE <b>DH</b> PMIK
ipns_flass	TD <b>W</b> HAMSAE	EKWELAIRAY	NPAN.PRNRN	GYMAVEGKK	ANESFCYLNP	SFDAD <b>H</b> ATIK
		124	131 135			
ipns_emeni	AKTPT <b>H</b> EVNV	WPDETK <b>H</b> PGF	QDFAEQYYWD	VFGLSSA.LL	KGYALALGKE	ENFFARHFKP
ipns_pench	SKTPT <b>H</b> EVNV	WPDEKK <b>H</b> PGF	REFAEQYYWD	VFGLSSA.LL	RGYALALGKE	EDFFSRHFKK
ipns_cepac	EPTPM <b>H</b> EVNV	WPDEAK <b>H</b> PGF	RAFAEKYYWD	VFGLSSA.VL	RGYALALGRD	EDFFTRHSRR
ipns_strju	SETPM <b>H</b> EVNL	WPDEEK <b>H</b> PRF	RPFCEYYRQ	LLRLSTV.IM	RGYALALGRR	EDFFDEALAE
ipns_strcl	AGTPM <b>H</b> EVNL	WPDEER <b>H</b> PRF	RPFCEYYRQ	MLKLSTV.LM	RGLALALGRP	EHFFDAALAE
ipns_nocla	AGTPM <b>H</b> EVNS	WPDEEK <b>H</b> PSF	RPFCEYYWT	MHRLSKV.LM	RGFALALGKD	ERFFEPPELKE
ipns_strgr	SGTPM <b>H</b> EGNI	WPDEKR <b>H</b> QRF	RPFCEYYRD	VFSLSKV.LM	RGFALALGKP	EDFFDASLSL
ipns_strlp	AGTPM <b>H</b> EVNV	WPDEER <b>H</b> PDF	RSFGEQYYRE	VFRLSKVLLL	RGFALALGKP	EEFFENEVTE
ipns_flass	AGLPS <b>H</b> EVNI	WPDEAR <b>H</b> PGM	RRFYEAIFYSD	VFDVA.VL	RGFAIALGRE	ESFFERHFSM
			203	212 214		
ipns_emeni	DDTLASVVL.	IRYPYLDYP	EAAIKTAADG	TKLSFEW <b>H</b> ED	VSLITVLYQS	NVQNLQVETA
ipns_pench	EDALSSVVL.	IRYPYLNPI	PAAIKTAEDG	TKLSFEW <b>H</b> ED	VSLITVLYQS	DVANLQVEMP
ipns_cepac	DDTLSSVVL.	IRYPYLDYP	EPAIKTADG	TKLSFEW <b>H</b> ED	VSLITVLYQS	DVQNLQVKTP
ipns_strju	ADTLSSVSL.	IRYPYLEEYP	..PVKTGADG	TKLSFED <b>H</b> LD	VSMITVLYQT	EVQNLQVETV
ipns_strcl	QDSLSSVSL.	IRYPYLEEYP	..PVKTGPDG	QLLSFED <b>H</b> LD	VSMITVLFQT	QVQNLQVETV
ipns_nocla	ADTLSSVSL.	IRYPYLEDYP	..PVKTGPDG	EKLSFED <b>H</b> FD	VSMITVLYQT	QVQNLQVETV
ipns_strgr	ADTLASVTL.	IHYPYLEDYP	..PVKTGPDG	TKLSFED <b>H</b> LD	VSMITVLFQT	EVQNLQVETA
ipns_strlp	EDTLSCRSML	IRYPYLDYP	EAAIKTGPDG	TRLSFED <b>H</b> LD	VSMITVLFQT	EVQNLQVETV
ipns_flass	DDTLASVSL.	IRYPFLENYP	..PLKLGPDG	EKLSFEH <b>H</b> QD	VSLITVLYQT	AIPNLQVETA
			268			
ipns_emeni	AGYQDIEADD	TGYLINCOSY	MAHLTNYYK	API <b>H</b> RVKWN	AERQSLPFFV	NLGYSVIDP
ipns_pench	QGYLDIEADD	NAYLVNCGSY	MAHITNNYYP	API <b>H</b> RVKWN	EERQSLPFFV	NLGFNDTVQP
ipns_cepac	QGWQDIQADD	TGFLINCOSY	MAHITDDYYP	API <b>H</b> RVKWN	EERQSLPFFV	NLGWEDTIQP
ipns_strju	DGWQDIPRSD	EDFLVNCCTY	MGHITHDYFP	APN <b>H</b> RVKFIN	AERLSLPFFL	NAGHNSVIEP
ipns_strcl	DGWRDIPTSE	NDFLVNCGTY	MAHVTNDYFP	APN <b>H</b> RVKFIN	AERLSLPFFL	NGGHEAVIEP
ipns_nocla	DGWRDLPTSD	TDFLVNAGTY	LGHLTNDYFP	SPL <b>H</b> RVKFIN	AERLSLPFFL	HAGQHTLIEP
ipns_strgr	DGWQDLPTSG	ENFLVNCCTY	MGYLTNDYFP	APN <b>H</b> RVKFIN	AERLSLPFFL	HAGHTTVMET
ipns_strlp	DGWQSLPTSG	ENFLVNCCTY	LGYLTNDYFP	APN <b>H</b> RVKYVN	AERLSLPFFL	HAGQNSVMKP
ipns_flass	EGYLDIPVSD	EHFLVNCCTY	MAHITNGYYP	APV <b>H</b> RVKYIN	AERLSIPFFA	NLSHASAIDP
ipns_emeni	FDPREP....	.NGKSDREPL	SYGDYLNQGL	VSLINKNGQT		
ipns_pench	WDPSKE....	.DGKTDQRPI	SYGDYLNQGL	VSLINKNGQT		
ipns_cepac	WDPAKADGA	KDAADKPAI	SYGEYLQGL	RGLINKNGQT		
ipns_strju	FVPEGA....	AGTVK.NPTT	SYGEYLQHGL	RALIVKNGQT		
ipns_strcl	FVPEGA....	SEEVN.NEAL	SYGDYLNQGL	RALIVKNGQT		
ipns_nocla	FFPDGA....	PEGKQNEAV	RYGDYLNHGL	HSLIVKNGQT		
ipns_strgr	FSPEDT....	RG.KELNPPV	RYGDYLNQGL	NALIAKNGQT		
ipns_strlp	FHPEDT....	.GDRKLNPAV	TYGEYLQEGF	HALIAKNVQT		
ipns_flass	FAP.PP....	YAPPGNPTV	SYGDYLNQGL	LDLIRANGQT		

FIGURE 1: Multiple sequence alignment of 10 bacterial and fungal IPNS primary amino acid sequences. Sources are, in descending order, *Aspergillus nidulans*, *Penicillium chrysogenum*, *Cephalosporium acremonium*, *Flavobacterium* sp., *Lysobacter lactamgenus*, *Streptomyces jumonjinensis*, *Streptomyces clavuligerus*, *Nocardia lactamdurans*, *Streptomyces griseus*, and *Streptomyces lipmanii*. Sequences were compiled from the SWISSPROT and PIR protein data bases. Numbers shown above the aligned sequence are the positions of conserved histidines (H) and aspartic acids (D) in IPNS sequences (numbering according to *S. jumonjinensis* IPNS).

conserved in all sequences (Figure 1). We replaced each of the conserved histidines and aspartic acids with alanines in the IPNS of *Streptomyces jumonjinensis* and analyzed the biochemical properties of the recombinant mutant proteins.

Our results identify two histidines and one aspartic acid that are essential for enzymatic activity. Furthermore, sequence analysis of IPNS and other non-heme Fe(II)-dependent dioxygenases reveals that all possess a common motif which

Table 1: Mutagenic Oligodeoxynucleotides Used in the Preparation of His → Ala and Asp → Ala Substitution Mutants of *S. jumonjinensis* Isopenicillin N Synthase

amino acid position	oligodeoxynucleotide sequence <sup>a</sup>			sequencing vector <sup>b</sup>	codon change
His48	5'-ACGTCCACACC	<u>GGC</u>	GTTCGACGCGTAG-3'	mp19	GTG → GGC
His63	5'-GCTCATGTTCCG	<u>GGC</u>	GAACCTCGTTTCAC-3'	mp19	GTG → GGC
His114	5'-TTGATCATCGG	<u>GGC</u>	GTCGTCGGAGAAC-3'	mp19	GTG → GGC
His124	5'-GAGGTTACCTC	<u>GGC</u>	CATCGGGGTCTC-3'	mp19	GTG → GGC
His135	5'-CCGGAACCGCGG	<u>GGC</u>	CTTCTCCTCGTC-3'	mp19	GTG → GGC
His212	5'-AGCTTCGAGGAC	<u>GCG</u>	CTGGACGTCTCGATG-3'	mp18	CAT → GCG
His268	5'-CCGGCCCCGAAC	<u>GCC</u>	CGGGTGAAGTTC-3'	mp18	CAC → GCC
Asp14	5'-CGGCGAGAT	<u>GGC</u>	GATGGTCGG-3'	mp19	GTC → GGC
Asp113	5'-GATCATCGGGTG	<u>GGC</u>	GTCGGAGAACGAC-3'	mp19	GTC → GGC
Asp131	5'-CTTCTCCTC	<u>GGC</u>	CGGCCAGAG-3'	mp19	GTC → GGC
Asp203	5'-CTCGGTACC	<u>GGC</u>	CGCGCCCCG-3'	mp19	GTC → GGC
Asp214	5'-GACCATCTG	<u>GCC</u>	GTCTCGATG-3'	mp18	GAC → GCC

<sup>a</sup> Altered codons are underlined. <sup>b</sup> For the M13mp19 sequencing vector the antisense codons are shown.

in IPNS contains the identical histidine and aspartic acid residues essential for function. On the basis of these observations we have been able to locate three of the four endogenous ligands proposed to form the IPNS ferrous active site. A partial account of these findings was presented in a poster at the International Symposium on the Genetics of Industrial Microorganisms, Montreal, June 1994. The genetic and sequence analysis of the *Streptomyces* IPNS active site presented here agrees with and complements that determined from the crystal structure of the *Aspergillus nidulans* IPNS that was reported while this paper was in preparation (Roach et al., 1995).

## MATERIALS AND METHODS

**Bacterial Strains and Plasmids.** Construction of the expression plasmid pOL-18 containing the IPNS gene of *S. jumonjinensis* NRRL 5741 under the control of an inducible T7 promoter was previously described (Landman et al., 1991). Expression of wild-type and mutant recombinant IPNS genes was carried out in *Escherichia coli* BL21(DE3) *plysS*. DNA manipulations were performed in *E. coli* TG1 (Sambrook et al., 1989).

**Site-Directed Mutagenesis.** Restriction fragments *Xba*I–*Asp*718 (0.65 kb) and *Asp*718–*Eco*RI (0.85 kb) conveniently divide the *S. jumonjinensis* IPNS gene and its flanking regions for the purpose of mutant construction. These were cloned into the single-stranded M13mp18 and M13mp19 vectors (Yanisch-Perron et al., 1985). Site-directed mutagenesis was performed according to the procedure of Eckstein (Sayers & Eckstein, 1991) using the Amersham Sculptor mutagenesis kit (Amersham International). The mutagenic oligonucleotides used in this work to change histidine and aspartic acid residues to alanine residues are shown in Table 1. The appropriate mutagenized region was sequenced by the dideoxy chain-termination method (Sanger et al., 1977) to verify the specific nucleotide change, exchanged for the wild-type region in pOL-18, and sequenced again to confirm the alteration in the expression plasmid.

**Expression.** Growth of transformants containing wild-type and mutant IPNS genes in the pOL-18 expression plasmid, and induction of synthesis of IPNS, has been described elsewhere (Landman et al., 1991). Cultures were induced with 0.4 mM isopropyl  $\beta$ -thiogalactopyranoside (IPTG) for 2 h at 37 °C and produced IPNS almost exclusively in the form of insoluble inclusion bodies; cultures grown for 3 h

at 30 °C in the presence of 0.01 mM inducer made small amounts of IPNS in the soluble form.

**Enzyme Purification.** Soluble IPNS preparations were obtained from the insoluble fraction of disrupted cells by denaturing in 5 M urea and refolding as previously reported (Landman et al., 1991). Wild-type and mutant IPNS preparations made in this way are referred to throughout in this article as solubilized IPNS and were approximately 95% pure, as judged by SDS–polyacrylamide gel electrophoresis. Soluble, crude IPNS from the supernatant fraction of cells induced at 30 °C was used without purification.

**Enzyme Assay and Kinetics.** The specific activities of unpurified soluble IPNS and solubilized IPNS from inclusion bodies were determined by an agar plate assay using *Micrococcus luteus* ATCC 381 as indicator (Jensen et al., 1982a). Kinetic constants  $K_m$  and  $k_{cat}$  were determined with purified solubilized enzyme preparations by following the consumption of ACV substrate and the appearance of isopenicillin N by HPLC (Jensen et al., 1982b). The standard reaction contained, in a final volume of 40  $\mu$ L, 0.69 mM ACV, 4 mM ascorbate, 4 mM DTT, 0.16 mM FeSO<sub>4</sub>, and up to 0.05 mM apoenzyme. Incubations were carried out for 20 min at 25 °C and terminated by addition of an equal volume of methanol. Conversion of ACV to isopenicillin N by *S. jumonjinensis* IPNS in the standard assay was linear with respect to time for at least 30 min in the conditions employed, as previously found with the *Streptomyces clavuligerus* enzyme (Jensen et al., 1982b) and in contrast to the rapid loss of activity reported for the *Cephalosporium acremonium* IPNS and ascribed to product inhibition (Perry et al., 1988). The data reported in Table 2 for the specific activity and kinetic constants of wild-type and mutant IPNSs are the average results of at least three experiments. The specific activities of the crude and solubilized wild-type preparations of IPNS were 29 and 530 nmol of isopenicillin N min<sup>-1</sup> (mg of protein)<sup>-1</sup>, respectively. IPNS concentrations were determined using the extinction coefficient at 280 nm (35 mM<sup>-1</sup> cm<sup>-1</sup>) calculated from the known amino acid composition; otherwise protein concentrations were determined with Coomassie blue using bovine serum albumin as standard (Bradford, 1976).

**Spectroscopy.** CD measurements were made in 0.1-mm cells in a Jasco J-500C spectropolarimeter operated at a scan rate of 20 nm/min in the range 190–270 nm. Protein samples were prepared in 50 mM Tris·HCl (pH 8.0) at a concentration of 0.0071 mM.

Table 2: Kinetic Parameters of IPNS His → Ala and Asp → Ala Mutants

IPNS mutant <sup>a</sup>	relative specific activity <sup>b</sup>		$K_m$ (mM)	$k_{cat}$ (min <sup>-1</sup> )	$k_{cat}/K_m$ (mM <sup>-1</sup> min <sup>-1</sup> )
	solubilized	soluble			
wild type	1.0	1.0	0.4	38.8	96.9
His48	0.16	0.23	0.56	7.5	13.4
His63	0.31	0.30	1.0	14.2	14.2
His114	0.28	0.18	0.85	12.5	14.7
His124	0.48	0.36	0.84	32.1	38.1
His135	0.22	0.17	0.59	11.7	19.8
His212	<0.007	<0.007	nm <sup>c</sup>	nm	nm
His268	<0.003	<0.007	nm	nm	nm
Asp14	0.05	0.03	0.86	0.56	0.7
Asp113	0.63	0.84	0.45	23.8	52.8
Asp131	0.68	0.70	0.48	36.3	75.5
Asp203	0.32	0.11	0.91	12.3	13.5
Asp214	<0.004	<0.007	nm	nm	nm

<sup>a</sup> Recombinant proteins made in *E. coli* from a T7-based expression system; see Materials and Methods. <sup>b</sup> Specific activities of solubilized IPNS preparations determined by bioassay and by HPLC assay; specific activities of crude soluble IPNS preparations determined by bioassay, see Materials and Methods. Values reported are averages based on at least three experiments; individual measurements varied about these values by no more than  $\pm 25\%$ . <sup>c</sup> nm: not measurable.

**Sequence Analysis.** BLASTP (Altschul et al., 1990) and PROFILE (Feng & Doolittle, 1987) searches were used to find the primary sequences of non-heme Fe(II)-containing dioxygenases in the PIR, Swissprot and Genbank databases. A total of 52 nonredundant protein sequences were extracted and aligned with the PileUp program of the GCG package (Devereux et al., 1984). This multiple sequence alignment was manually edited to show the potential iron-binding residues present in all the aligned sequences. Numbering of amino acid residues shown in the alignments reported in this paper is that of *S. jumonjinensis* IPNS (Figure 2).

## RESULTS

**Expression of Mutant IPNS Genes.** *S. jumonjinensis* IPNS contains 13 histidines and 23 aspartic acids. Each of the seven conserved histidines and five conserved aspartic acids in the bacterial and fungal IPNS proteins (Figure 1) is, according to spectroscopic studies, a potential candidate as an iron ligand and was exchanged for alanine by site-directed mutagenesis. Figure 2 shows the effect of the point mutations on synthesis of recombinant proteins in *E. coli*. All of the mutant proteins were made to about the same extent as judged by SDS gel electrophoresis of solubilized IPNS preparations.

**Biochemical Analysis of Mutant IPNS Enzymes.** Recombinant IPNS made with the T7 expression system was almost entirely insoluble. Highly purified solubilized IPNS was recovered after centrifugation of cell sonicates, denaturation in urea, and renaturation as previously described for the wild-type protein (Landman et al., 1991). Biochemical characterization of wild-type and mutant IPNS enzymes prepared in this way is reported in Table 2. Specific activities of the mutant enzymes have been normalized to that of the wild-type enzyme. The corresponding specific activities for the untreated soluble IPNS mutant enzymes present in the cell supernatant fraction are shown alongside. Comparison of the two sets of data shows that the relative specific activity of each of the mutant enzymes is qualitatively the same for the purified solubilized preparation and the crude soluble supernatant fraction and justifies use of the solubilized IPNS

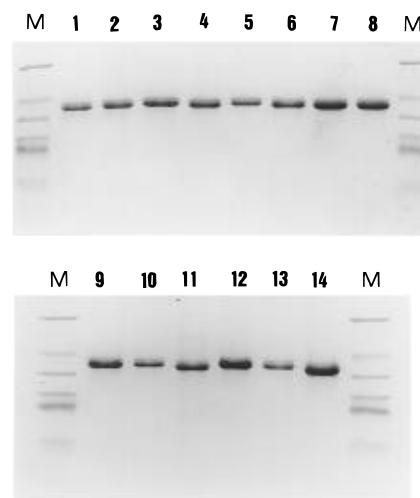


FIGURE 2: SDS-polyacrylamide gel electrophoresis of *S. jumonjinensis* wild type and mutant IPNS. The recombinant proteins were prepared as described in Materials and Methods. Upper panel: lanes 1–8 refer to, in ascending order, wild type and His48, His63, His114, His124, His135, His212, and His268 alanine-substituted mutant proteins. Lower panel: lanes 9–14 refer to, in ascending order, wild type and Asp14, Asp113, Asp131, Asp203 and Asp214 mutant proteins. M, molecular mass standards: 20, 24, 29, 36, 45, and 66 kDa.

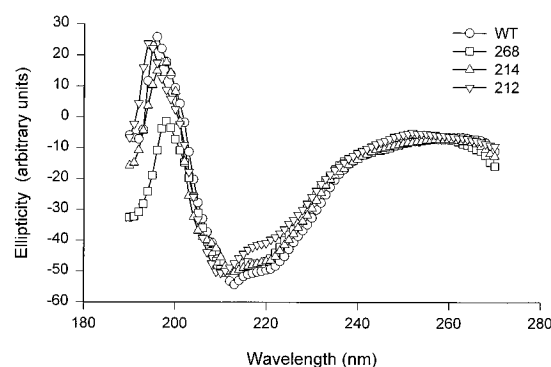


FIGURE 3: CD spectra of *S. jumonjinensis* wild type and His212, Asp214, and His268 alanine-substituted mutant proteins.

preparations for more detailed kinetic studies (see below). The mutant enzymes fall into two classes. His212, His268, and Asp214 have no detectable activity in either the HPLC or bioassay; His48, His63, His114, His124, and His135 and Asp14, Asp113, Asp131, and Asp203 possess 5–68% the specific activity of wild type.

Physical characterization of the wild-type and the three inactive enzymes, His212, Asp214, and His268, by CD spectroscopy showed no significant difference in conformation between the wild-type and mutant proteins (Figure 3).

Kinetic parameters  $K_m$  and  $k_{cat}$  for 9 of the 12 IPNS mutant enzymes and for wild type are presented in Table 2. It was not possible to determine these constants for the three inactive enzymes, His212, His268, and Asp214.  $K_m$  values for the partially inactive enzymes were not appreciably different from that of the wild type, indicating that in these mutant enzymes the histidine and aspartic acid replacements did not substantially affect substrate binding. Values of  $k_{cat}$  for 8 of the 9 partially inactive IPNS enzymes were 1.1–5.2-fold less than that of wild type; the  $k_{cat}$  of one enzyme, Asp14, was much more severely affected than the others and was approximately 70-fold less than wild type.

	block I	block II	block III
	41 48	212 214	268 277
HYD_STRCL	... ECVTGMGVFYLTYG. AGDK ...	PRRMAPHYDLSIITFIHQTP ...	ALPAPRHHVSPGAGMREGSDRTSSVFFLR ...
EXP_STRCL	... RCLRDKGFLYLTDCGL. TDT ...	PLRMAPHYDLSMTLTIQQT ...	QVKAPRHHVAAPRRDQIAGSSRTSSVFFLR ...
EXP/HYD_CEPAC	... EAVTTKGIFYLTESGLVDD ...	PLRMGPHYDLSTITLVHQT ...	KVKAPKHRVKSPPGRDQVGSRTSSVFFLR ...
IPNS_STRJU	... KAARGSGFFYASNHGVDVQL ...	KLSEFEDHLDVSMITVLVQTE ...	YFPAENHRV ... KFNAERLSLPFFLN ...
ANS_MAIZE	... AAAAGDGMVHIAHGIPAE ...	AVGVEAHTDVSALSFIHNG ...	RYTSVLHRLGV ... NRE. AVRISWVVFCE ...
FS_PETHY	... DASKEWGIFQLINHGIPDEA ...	ALGVVAHTDMSYITILVNE ...	KYKSVYHRTTV ... NKD. KTRMSWVFLE ...
FL3H_HORVU	... AACEDWGIFQVIDHGVDADL ...	TLGLKRHTDPGTITLLQDL ...	RFKNADHQAVV ... NGESS. RLSIATFQN ...
ACCO_PETHY	... DACENWGFFELVNHGIPREV ...	IKGLRAHTDAGGIILFQDD ...	KYKSVHHRVIA ... QKDGA. RMSLASFYN ...
E8_LYCES	... DASEKWGFFQVNVHGIPTSV ...	TMGTIQHTDIGFVTILLQDD ...	KYLSVEHRAIS ... NNVG. SRMSITCFFG ...
HY6H_HYONI	... KACQDFGLFQVINHGFPPEL ...	TLGSGGHYDGNLIT. LLQDD ...	KFEGSIHRVVT ... DPTRD. RVSIATLIG ...
GH_CUCMA	... EACRQHGIFFVVNHGVDIEM ...	VLGTGPHTDPTSVTILHQDP ...	IYKGCIHRAVV ... NSMNA. RKSIAFFLC ...

FIGURE 4: **HisXAsp(53–57)XHis** motif in IPNS and other non-heme ferrous iron-containing dioxygenases. Multiple sequence alignment of three blocks of amino acids in *S. jumonjinensis* with the corresponding sequences from representative members of 10 other classes of Fe(II)-dependent dioxygenases. Numbering is according to *S. jumonjinensis*. Enzyme designations, sources, and data bank references are as follows, in descending order. HYD\_STRCL: deacetylcephalosporin C synthase from *S. clavuligerus*, SW:CEFE\_STRCL. EXP\_STRCL: deacetoxycephalosporin C synthase from *S. clavuligerus*, PIR2:A39204. EXP/HYD\_CEPAC: expandase/hydroxylase from *C. acremonium*, PIR2:A29711. IPNS\_STRJU: isopenicillin N synthase (IPNS) from *S. jumonjinensis*, SW:IPNS\_STRJU. ANS\_MAIZE: A2 gene from *Z. mays*, SW:ANS\_MAIZE. FS\_PETHY: flavonol synthase from *P. hybrida*, PIR3:S33510. FL3H\_HORVU: flavanone-3-hydroxylase from *H. vulgare*, SW:FL3H-HORVU. ACCO\_PETHY: ethylene-forming enzyme from *P. hybrida*, SW:ACCI\_PETHY. E8\_LYCES: 1-aminocyclopropane-1-carboxylic acid oxidase homolog from *L. esculentum*, SW:ACCH\_LYCES. HY6H\_HYONI: hyoscyamine 6- $\beta$ -hydroxylase from *H. niger*, SW:HY6H\_HYONI. GH\_CUCMA: gibberellin 20-oxidase from *C. maxima*, GB\_CMGIB.

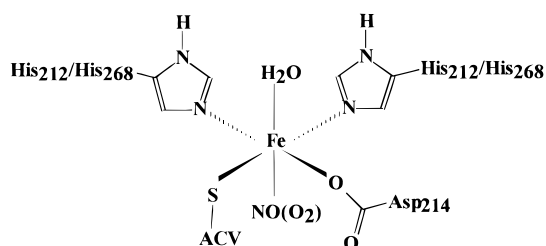


FIGURE 5: Assignment of histidine and aspartic acid endogenous ligands in the IPNS ferrous active site. Numbers refer to the positions of histidine and aspartic acid residues in *S. jumonjinensis* IPNS that are essential for catalytic activity. The spatial arrangement of His212 and His268 ligands with respect to ACV in the equatorial plane is not unambiguously determined.

**Sequence Analysis of IPNS and Other Non-Heme Fe(II) Dioxygenases.** A total of 52 nonredundant primary sequences were extracted with the use of PROFILE and BLASTP searches from the Swissprot, PIR, and Genbank databases. These include 10 IPNS enzymes, several other  $\beta$ -lactam biosynthetic enzymes, including three hydroxylases (deacetoxycephalosporin C synthase), three expandases (deacetylcephalosporin C synthase), and one expandase/hydroxylase, 18 1-aminocyclopropane-1-carboxylic acid (ACCO) oxidases, nine flavanone 3 $\beta$ -hydroxylases, one flavonol synthase, two E8 proteins, one gibberellin A20 oxidase, one hyoscyamine 6 $\beta$ -hydroxylase, and three anthocyanidin hydroxylases. All of the enzymes use Fe(II) and ascorbate and all but IPNS and ACCO require 2-oxoglutarate. The sequences were aligned with the PileUp program. Figure 4 is an abridged alignment to show the five residues that are totally conserved: Gly41, His212, Asp214, His268, and Arg277 (numbering according to *S. jumonjinensis* IPNS). The alignment is partitioned such that segment I contains Gly41, segment II contains His212 and Asp214, and segment III contains His268 and Arg277. With the exception of the  $\beta$ -lactam biosynthetic enzymes, expandase, hydroxylase, and expandase/hydroxylase and one bacterial ACCO, His48 is also conserved in all sequences (segment I). Inspection of Figure 4 shows that all the dioxygenases possess the motif **HisXAsp(53–57)XHis**. In IPNS it contains each of the two histidines, His212 and His268, and the one aspartic acid, Asp214, that are essential for enzymatic activity.

## DISCUSSION

Spectroscopic studies of IPNS have led to a physical model for the coordination environment of the ferrous active site [reviewed in Cooper (1993)] in which the metal center in the holoenzyme contains three endogenous histidines, an unsymmetrically chelated endogenous aspartate carboxylate, and a solvent molecule. A refinement of that model proposes that formation of the Fe(II) IPNS–ACV enzyme–substrate complex occurs through displacement of the H<sub>2</sub>O; subsequent binding of dioxygen to that complex results in exchange of the axial histidine ligand by a solvent molecule, creating an Fe(II) center with sites for two histidines, one aspartic acid, and the cysteinyl thiolate from ACV (Figure 5). According to the above, two histidines and one aspartic acid are critical features of the IPNS ferrous active site. The genetic analysis of *S. jumonjinensis* IPNS presented in this work shows that alanine substitutions of the amino acids His212, Asp214, and His268 result in enzymes with no measurable activity, whereas appreciable activity is retained in all other histidine and aspartic acid replacements. CD spectroscopy studies demonstrate that these substitutions do not cause any significant conformational changes. We propose, therefore, that His212, Asp214, and His268 correspond to three of the four endogenous ligands postulated from physical studies to determine the IPNS ferrous active site (Figure 5). Our results agree with and considerably extend those of a recent genetic analysis of the fungal *C. acremonium* IPNS and reported while this work was in progress; this study was confined to showing that His272 (equivalent to His268 of *S. jumonjinensis*) is essential for activity (Tiow-Suan & Tan, 1994). The reduced activity of the remaining mutant enzymes, with the possible exception of the extremely defective Asp14 mutant, may reflect general effects of the alanine substitutions on protein structure.

Support for the above assignments comes from comparative primary amino acid sequence analysis of a broad group of non-heme Fe(II)-dependent dioxygenases. All of the enzymes activate dioxygen and have a strict requirement for ferrous ions and ascorbate, and several require 2-oxoglutarate. They carry out hydroxylations, desaturations, and oxidative cyclizations in secondary metabolic processes, including reactions involved in the biosynthesis of penicillins and cephalosporins in bacteria and fungi (Baldwin & Abraham,

1988; Cohen et al., 1990), the production of flavonoids (Britsch et al., 1981), alkaloids (Hashimoto & Yamada, 1986), and hormones (Hedden & Graebe, 1982) in plants, and the modification of proline and lysine residues in collagens in vertebrates (Kivirikko et al., 1989). A common general mechanism appears to unite these diverse reactions, and consequently analysis of the primary amino acid sequences of these dioxygenases was carried out to search for functional and structural motifs.

Multiple sequence alignment of 11 representative members of 52 non-heme Fe(II)-containing dioxygenases (Figure 4) establishes that just two histidines and one aspartic acid are entirely conserved. It is highly significant, therefore, that in IPNS these two histidines, His212 and His268, and one aspartic acid, Asp214, are precisely the ones essential for activity. We conclude from the sequence analysis that the assignment of the IPNS histidine and aspartic acid endogenous ligands in the ferrous active site, depicted in Figure 4 and based on the mutagenesis analysis alone, is highly probable and corresponds to three of the four ligands postulated from spectroscopic studies of IPNS. Furthermore, we propose that the motif **HisXAsp(53–57)XHis** determines a structural and functional element common to this class of non-heme Fe(II) dioxygenases and critical to their mechanism of action. We note also that just two other amino acid residues, Gly41 and Arg277, are conserved in all 52 dioxygenases (*S. jumonjinensis* numbering). The Arg277 residue has been suggested to be involved in 2-oxoglutarate and/or ascorbate binding (Britsch et al., 1992), but no possible function has been ascribed for Gly41. Similar but less definitive conclusions were previously reached from alignment of non-heme Fe(II) dioxygenases (Matsuda et al., 1991; Myllyla et al., 1992; Britsch et al., 1993) but could not be substantiated through lack of genetic or structural evidence. We have used the **HisXAsp(53–57)XHis** motif to screen the protein data bases and found, in addition to the above 52 proteins, four more proteins (or open reading frames), two from barley, one from *Arabidopsis thaliana*, and one from *Schizosaccharomyces pombe* that have no known function. These were not included in the above sequence analysis. We anticipate that the activity of these proteins will require ferrous iron.

EPR studies of Co(II) IPNS and Cu(II) IPNS–ACV demonstrate that two of the three endogenous histidine ligands, and the ACV substrate, lie in the equatorial plane of the hexacoordinated metal center (Jiang et al., 1991). EPR studies also show that nitric oxide, frequently used as an analog for O<sub>2</sub> with heme proteins and dioxygenases (Arciero & Lipscomb, 1986), occupies an axial position in the coordination center (Chen et al., 1989). Accordingly, in Figure 5 we have drawn the His212 and His268 endogenous ligands and the ACV exogenous ligand in the equatorial plane, and the exogenous O<sub>2</sub> ligand in the axial state. We also place the aspartic acid endogenous ligand, Asp214, in the equatorial plane because a similar geometry exists for the three histidines and one aspartic acid in the active site of non-heme iron superoxide dismutase (Stoddard et al., 1990). Moreover, secondary structure analysis of IPNS (data not shown) suggests that His212 and Asp214 occur in a region of extended sheet structure which is compatible with the two ligands adopting a *cis* configuration in the equatorial plane. This arrangement is supported by models based on typical protein secondary structure from X-ray data which

show that the motif HisXAsp in a  $\beta$ -strand can form energetically stable chelating sites for Ni(II) and Cu(II) (Arnold & Zhang, 1994).

Spectroscopic studies indicate that four endogenous ligands, three histidines and one aspartic acid, define the metal center of the IPNS holoenzyme and its substrate complex and that one of the histidines is displaced by a solvent molecule on binding of molecular oxygen (nitric oxide) (Ming et al., 1991). Biochemical analysis of the seven histidine-deficient mutants described in this work failed to identify the third histidine ligand. Indeed, it is not clear what would be the consequences for enzymatic activity on replacing that histidine ligand with alanine. Each of the five histidine mutants, His48, His63, His114, His124, and His135, has substantial activity, although His48 is more defective than the others. The multiple sequence alignment of non-heme Fe(II)-dependent dioxygenases shows that in 52 sequences six lack a histidine residue at the position equivalent to 48 in *S. jumonjinensis* IPNS. Five of the exceptions are noteworthy because they, like IPNS, are enzymes involved in the  $\beta$ -lactam biosynthetic pathway, deacetoxycephalosporin synthase and deacetylcephalosporin synthase. The former is the ring expansion enzyme, and the latter carries out ring hydroxylation (Cooper, 1993). The other example is the ethylene-forming enzyme of *Pseudomonas syringae* pv. *phaseolicola* 1-aminocyclopropane-1-carboxylic acid oxidase (Fukuda et al., 1993), whereas all other members of this class of oxidases contain the equivalent His48. On the basis of these findings it is possible that the spectroscopic studies may have misidentified the fourth endogenous ligand in the metal center—that it is not a histidine and this would account for our not identifying it.

Compelling evidence for the conclusions reached in the above discussion has very recently come from the studies of Roach et al. (1995) who have determined the 3D structure of the *A. nidulans* IPNS. Crystals of IPNS were prepared with manganese replacing iron at the active site. The structure of the active site reveals four protein ligands attached to the metal ion, three of which correspond precisely to those identified by our genetic analysis as the endogenous ligands of the ferrous active site in the *Streptomyces* IPNS. The fourth ligand is the penultimate glutamine residue in the IPNS molecule and is the ligand that is likely to be displaced on substrate binding. The genetic analysis described in this paper complements that derived from the crystal structure and indicates that the coordination environment of the manganese and iron atoms in the fungal and bacterial enzymes, respectively, is essentially the same.

In view of the above results, we attempted to determine by direct iron binding experiments whether elimination of any one of the His212, Asp214, and His268 ligands affects iron binding. When an equimolar amount of ferrous iron was incubated with the wild-type and mutant proteins, all bound essentially the same amount of iron, which, in the case of the wild-type protein, was estimated to be that needed for maximal enzyme activity (Chen et al., 1989). At higher levels of iron, from 1 to 10 equiv/mol of enzyme, both the wild-type and mutant proteins (only the His268 mutant was tested) bound increasing amounts of iron up to 3 equiv/mol of protein, indicating the existence in the *Streptomyces* IPNS of two additional iron-binding sites. Roach et al. (1995) also found evidence for an additional metal binding site in their crystal studies of the *A. nidulans* IPNS. Interestingly,

increasing the iron concentration in reactions carried out with the His268 mutant did not result in the recovery of detectable enzyme activity. These observations suggest that the mutant proteins bind iron to a similar extent as the wild type. Presumably, the coordination environment at the ferrous site in the mutant proteins must differ in some subtle way from that of the wild type to prevent IPNS chemistry.

We are currently involved in a collaborative effort to elucidate the crystal structure of the *S. jumonjinensis* IPNS and its substrate complex and to characterize the role of the endogenous ligands in the ferrous active site and understand the mechanism of IPNS catalysis. We predict that the structural elements and catalytic properties of the IPNS metal center will prove to be similar to that to be determined among other non-heme Fe(II) dioxygenases.

## ACKNOWLEDGMENT

We thank Drs. C. T. Schofield and P. L. Roach and Professor J. E. Baldwin for kindly communicating their results on the crystal structure of IPNS prior to publication, and Dr. Vlad Brunfeld for performing CD measurements.

## REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410.
- Arciero, D. M., & Lipscomb, J. D. (1986) *J. Biol. Chem.* 261, 2170–2178.
- Arnold, F. M., & Zhang, J. (1994) *Trends Biotechnol.* 12, 189–192.
- Baldwin, J. E. (1989) in *Recent Advances in the Chemistry of  $\beta$ -Lactam Antibiotics* (Bentley, P. H., & Southgate, R., Eds.) Chapter 1, Royal Society of Chemistry, London.
- Baldwin, J. E. & Abraham, E. P. (1988) *Nat. Prod. Rep.* 5, 129–145.
- Baldwin, J. E. & Bradley, M. (1990) *Chem. Rev.* 90, 1079–1088.
- Bradford, M. M., (1976) *Anal. Biochem.* 72, 248–254.
- Britsch, L., Ruhnau-Brich, B., & Forkmann, G. (1992) *J. Biol. Chem.* 267, 5380–5387.
- Britsch, L., Dedio, J., Saedler, H., & Forkmann, G. (1993) *Eur. J. Biochem.* 217, 745–754.
- Chen, V. J., Orville, A. M., Harpel, M. R., Frolik, C. A., Surerus, K. K., Munck, E., & Lipscomb, J. D. (1989) *J. Biol. Chem.* 264, 21677–21681.
- Cohen, G., Shiffman, D., Mevarech, M., & Aharonowitz, Y. (1990) *Trends Biotechnol.* 8, 105–111.
- Cooper, R. D. (1993) *Bioorg. Med. Chem.* 1, 1–17.
- Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res.* 12, 387–395.
- Feng, D. F., & Doolittle, R. F. (1987) *J. Mol. Evol.* 25, 351–360.
- Fukuda, H., Ogawa, T., Ishihara, K., Fujii, T., Nagahama, K., Omata, T., Inoue, Y., Tanase, S., & Morino, Y. (1992) *Biochem. Biophys. Res. Commun.* 188, 826–832.
- Hashimoto, T., & Yamada, Y. (1986) *Plant. Physiol.* 81, 619–625.
- Hedden, P., & Graebe, J. E. (1982) *J. Plant Growth Regul.* 1, 105–116.
- Ingraham, L. L., & Meyer, D. L. (1985) in *Biochemistry of Dioxygen* (Frieden, E., Ed.) pp 139–246, Plenum Publishing Co., New York.
- Jensen, S. E., Westlake, D. W. S., & Wolfe, S. (1982a) *J. Antibiot.* 35, 1026–1032.
- Jensen, S. E., Westlake, D. W. S., & Wolfe, S. (1982b) *J. Antibiot.* 35, 483–490.
- Jiang, F., Peisach, J., Ming, L. J., Que, L., Jr., & Chen, V. J. (1991) *Biochemistry* 30, 11437–11445.
- Kivirikko, K. I., Myllyla, R., & Pihlajaniemi, T. (1989) *FASEB J.* 3, 1609–1617.
- Landman, O., Shiffman, D., Av Gay, Y., Aharonowitz, Y., & Cohen, G. (1991) *FEMS Microbiol. Lett.* 68, 239–244.
- Matsuda, J., Okabe, S., Hashimoto, T., & Yamada, Y. (1991) *J. Biol. Chem.* 266, 9460–9464.
- Ming, L. J., Que, L., Jr., Kriauciunas, A., Frolik, C. A., & Chen, V. J. (1990) *Inorg. Chem.* 29, 1111–1112.
- Ming, L. J., Que, L., Jr., Kriauciunas, A., Frolik, C. A., & Chen, V. J. (1991) *Biochemistry* 30, 11653–11659.
- Myllyla, R., Gunzler, V., Kivirikko, K. I., & Kaska, D. D. (1992) *Biochem. J.* 286, 923–927.
- Orville, A. M., Chen, V. J., Kriauciunas, A., Harpel, M. R., Fox, B. G., Munck, E., & Lipscomb, J. D. (1992) *Biochemistry* 31, 4602–4612.
- Perry, D., Abraham, E. P., & Baldwin, J. E. (1988) *Biochem. J.* 255, 345–351.
- Que, L., Jr. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., Ed.) Chapter 6, VCH, New York.
- Randall, C. R., Zang, Y., True, A. E., Que, L., Jr., Charnock, J. M., Garner, C. D., Fujishima, Y., Schofield, C. J., & Baldwin, J. E. (1993) *Biochemistry* 32, 6664–6673.
- Roach, P. L., Clifton, I. T., Fulop, V., Harlos, K., Barton, G. J., Hadjdu, J., Anderson, I., Schofield, C. I., & Baldwin, J. E. (1995) *Nature* 375, 700–704.
- Robinson, J. A. (1988) *Chem. Soc. Rev.* 17, 383–452.
- Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Sambrook, J., Fritsch, E. F., & Maniatis, T., Eds.) pp 1.25–1.31, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467.
- Sayer, J. R., & Eckstein, F. (1991) in *Directed Mutagenesis: A Practical Approach* (McPherson, M. J., Ed.) pp 49–69, IRL Press/Oxford University Press, New York and Tokyo.
- Scott, R. A., Wang, S., Eidsness, M. K., Kriauciunas, A., Frolik, C. A., & Chen, V. J. (1992) *Biochemistry* 31, 4596–4601.
- Stoddard, B. L., Howell, P. L., Ringe, D., & Petsko, G. A. (1990) *Biochemistry* 29, 8885–8893.
- Tiow Suan, S., & Tan, D. S. (1994) *FEMS Microbiol. Lett.* 120, 241–247.
- White, R. L., John, E. M., Baldwin, J. E., & Abraham, E. P. (1982) *Biochem. J.* 203, 791–793.
- Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene* 33, 103–119.

BI951534T