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ARTICLE *in* BIOPHYSICAL CHEMISTRY · SEPTEMBER 2000

Impact Factor: 1.99 · DOI: 10.1016/s0301-4622(00)00123-x · Source: PubMed

ELSEVIER

Biophysical Chemistry 86 (2000) 109–118

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Structure–function studies of the non-heme iron active site of isopenicillin *N* synthase: some implications for catalysis ☆

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Received 10 December 1999; accepted 7 January 2000

Abstract

Isopenicillin *N* synthase (IPNS) is a non-heme ferrous iron-dependent oxygenase that catalyzes the ring closure of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to form isopenicillin *N*. Spectroscopic studies and the crystal structure of IPNS show that the iron atom in the active species is coordinated to two histidine and one aspartic acid residues, and to ACV, dioxygen and H_2O. We previously showed by site-directed mutagenesis that residues His212, Asp214 and His268 in the IPNS of *Streptomyces jumonjinensis* are essential for activity and correspond to the iron ligands identified by crystallography. To evaluate the importance of the nature of the protein ligands for activity, His214 and His268 were exchanged with asparagine, aspartic acid and glutamine, and Asp214 replaced with glutamic acid, histidine and cysteine, each of which has the potential to bind iron. Only the Asp214Glu mutant retained activity, ~1% that of the wild type. To determine the importance of the spatial arrangement of the protein ligands for activity, His212 and His268 were separately exchanged with Asp214; both mutant enzymes were completely defective. These findings establish that IPNS activity depends critically on the presence of two histidine and one carboxylate ligands in a unique spatial arrangement within the active site. Molecular modeling studies of the active site employing the *S. jumonjinensis* IPNS crystal structure support this view. Measurements of iron binding by the wild type and the Asp214Glu, Asp214His and Asp214Cys-modified proteins suggest that Asp214 may have a role in catalysis as well as in iron coordination.

Keywords: Isopenicillin *N* synthase; Non-heme iron dioxygenase; Active-site protein ligands; Mutagenesis; *Streptomyces*

☆ Dedicated to Heini Eisenberg.

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0301-4622/00/$ - see front matter
PII: S0301-4622(00)00123-X

1. Introduction

Isopenicillin *N* synthase (IPNS) is a non-heme ferrous iron-dependent dioxygenase that mediates a key step in the biosynthesis of penicillin and cephalosporin antibiotics in bacteria and fungi [1]. IPNS catalyzes the oxidative ring closure of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to form isopenicillin *N* in a reaction in which molecular oxygen is completely reduced to water [2]. New insights into the mechanism of this remarkable reaction have come from recent studies on the structure of the iron coordination center and its role in catalysis. The crystal structure of the *Aspergillus nidulans* IPNS and its substrate complex [3,4] and a wealth of spectroscopic studies of IPNS (reviewed in [5]) are consistent with a picture in which the iron atom in the catalytically active species is attached to the tripeptide substrate, to three protein ligands — two histidines and one aspartic acid — and to ACV, dioxygen and a water molecule. A similar 2-His-1-carboxylate triad is found in the crystal structures of several other ferrous iron dioxygenases that catalyze very different reactions [6]. In each case the protein ligands are arranged in an array on one face of an octahedron, anchoring the iron atom in the active site while making accessible the remaining sites for binding of additional protein residues and/or exogenous ligands such as the substrate and cofactor.

In previous studies, we used site-directed mutagenesis to determine which of the conserved histidines and aspartic acids present throughout the microbial IPNSs are necessary for function [7]. We showed that residues His212, Asp214 and His268 in the IPNS of *Streptomyces jumonjinensis* are essential for activity and correspond to the active site iron–protein ligands identified from the *A. nidulans* IPNS crystal structure. A search of the protein sequence data banks showed that two conserved histidines and an aspartic acid (or glutamic acid) are common to a large family of non-heme ferrous iron oxygenases. A subset of these — including IPNS, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase which converts ACC to ethylene, and various other dioxygen-activating enzymes that require 2-oxoglutarate as a cofactor and which are involved in the production of secondary metabolites in bacteria, fungi and plants and in the modification of collagen — all contain these three residues in a characteristic sequence motif denoted His-X-Asp-(53-57)X-His [7]. Site-directed mutagenesis of the conserved histidine and aspartic acid residues of the IPNS of *Cephalosporium acremonium* [8,9], of several plant ACC oxidases, apple fruit [10], kiwi fruit [11] and tomato [12], a petunia flavanone 3β-hydroxylase [13] and a human prolyl 4-hydroxylase [14] has verified that these three residues are essential for catalysis in each of these enzymes. Chemical modification of histidine residues in ACC oxidase and some other members of this family support these conclusions [12,15,16].

While there is compelling evidence in the above dioxygen-activating enzymes that the histidine–carboxylate triad functions in coordinating the iron atom, facilitating binding of substrates, cofactors and O_2 (reviewed in [6]), it is not clear to what extent the nature and arrangement of the three endogenous ligands contribute to their catalytic activity. The study described here is an attempt to address this issue in IPNS using site-directed mutagenesis to modify the active site ligands in two ways: (i) by creating compositional mutants in which each of the three protein ligands was replaced with other amino acid residues which have the potential to bind iron but differ in their chemical and physical properties from the native ligand; and (ii) by creating spatial mutants in which the arrangement of the three protein ligands in the active site was cyclically permuted without changing their composition. We show that the enzymatic activity of IPNS is critically dependent on its possessing in its iron active site two histidine and one aspartic acid residues in a unique spatial arrangement.

2. Experimental procedures

2.1. Plasmids

Plasmid pOL18 containing the wild type *S. jumonjinensis* IPNS gene under the control of the phage T7 promoter was used for the construction

and expression in *E. coli* of mutant IPNS genes as previously described [7].

2.2. DNA manipulations and site-directed mutagenesis

Standard DNA manipulations procedures, the preparation of competent cells and transformation of *E. coli* were performed as described [17]. Site-directed mutagenesis was carried out using the Amersham Sculptor kit (Amersham, UK) as previously described [7]. Synthetic oligodeoxynucleotides were obtained from Biotechnology General (Rehovot, Israel) and designed so as to incorporate or destroy a new restriction site in the mutant region of the IPNS gene (Table 1). The sequence of modified IPNS genes was verified with the ABI Prism Automatic Sequencer (Perkin Elmer Biosystems, Foster City, CA, USA). IPNS single mutants constructed in this study were: H212D, H212N, H212Q; D214E, D214H, D214C; H268D, H268N, H268Q. The IPNS double mutant H212D, D214H was constructed using the DNA encoding D214H as the template; the double mutant D214H, H268D was constructed by combining the individual single mutant DNAs.

2.3. Expression of recombinant mutant proteins, purification and enzyme activity assay

Plasmid pOL18 derivatives with mutant IPNS genes were transformed into *E. coli* BL21/DE3 (p*lys*S) for expression of recombinant proteins. Briefly, cultures of transformants grown at 37°C in Luria Broth were induced with 0.4 mM isopropyl β-thiogalactopyranoside for 2 h, the cells harvested and disrupted by sonication and the insoluble protein denatured in urea and renatured as previously described [18]. The solubilized IPNS proteins were greater than 95% pure as judged from SDS–polyacrylamide gel electrophoresis. The activity of recombinant proteins was measured by following the conversion of ACV to isopenicillin *N* by reverse phase HPLC [19].

2.4. Iron binding

Quantitation of iron binding was performed employing a colorimetric assay [20] with modifications [21]. Wild type and mutant proteins were incubated with two equivalents of ferrous iron from an anaerobic solution of ferrous ammonium sulfate prepared in distilled water. Free iron was separated from the bound iron by chromatography on a P4 Biogel column. Protein concentrations were determined using the extinction coefficient at 280 nm, 35 mM^{-1} cm^{-1} [7].

2.5. Molecular modeling

The crystal structure of the *S. jumonjinensis* apo-IPNS and the IPNS-Fe(II)-δ-(L-α-aminoa-

Table 1
Oligonucleotides used to perform site-directed mutagenesis of the active site histidine and aspartic acid ligands of IPNS

Mutation	Oligonucleotide sequence[a]	Restriction site change
H212N	5′-CTGAGCTTCGAggac aAT CTGGACGTCTCG-3′	Loss of AvaII
H212D	5′-CTGAGCTTCGAGGAC gac ctggacgtcTCG-3′	Gain of DrdI
H212Q	5′-CTGAGCTTCGAGGAC cag ctgGACGTCTCG-3′	Gain of PvuII
D214C	5′-CGAGGACCATCTG tgc gtcTCGATGATC-3′	Loss of AatII
D214E	5′-CGAGGACCATCTG gaa gtcTCGATGATC-3′	Loss of AatII
D214H	5′-CGAGGACCATCTG cat gtcTCGATGATC-3′	Loss of AatII
H268N	5′-CCGGCCCCGAAC aac cgggtgAAGTTC-3′	Loss of DrdIII
H268D	5′-CCGGCCCCGAAc gat cgGGTGAAGTTC-3′	Gain of PvuI
H268Q	5′-CCGGCCCCGAAC cag cgggtgAAGTTC-3′	Loss of DrdIII

[a] The nucleotide changes that create the altered amino acid codons are shown underlined; the gain or loss of a restriction site that enables the wild type and mutant gene to be differentiated is shown in lowercase.

dipoyl)-L-cysteinyl-glycine (ACG) substrate–inhibitor complex (unpublished results) were used to model the structures of the D214E, D214C, D214H and H212D, D214H and D214H, H268D mutant proteins using the programs O [22], Quanta 96, (Molecular Simulations Inc, Burlington, MA, USA) and SETOR [23].

3. Results and discussion

3.1. Activity of compositional IPNS mutant proteins

Modified IPNS proteins were designed to replace the His212 and His268 ligands with asparagine, aspartic acid or glutamine, and the Asp214 ligand with glutamic acid, histidine or cysteine, each of which has the potential to bind ferrous iron. Wild type IPNS and the nine single compositional mutant proteins — H212N, H212D, H212Q, D214E, D214H, D214C, H268N, H268D, H268Q (and two double spatial mutant proteins referred to below) were highly overproduced employing the *E. coli*–T7 expression system and were produced mainly in an insoluble form. The recombinant wild type and mutant proteins were solubilized as previously described [7] and their mobility in SDS-polyacrylamide gel electrophoresis found to be indistinguishable (data not shown). With the sole exception of the D214E mutant protein, no measurable activity was found in any of the single mutant proteins (less than 0.2% the activity of wild type). Substitution of Asp214 by glutamic acid resulted in an enzyme with $\sim 1\%$ of the wild type activity. Table 2 shows the specific activity and apparent K_m and k_{cat} values for the wild type protein and the D214E mutant protein. The apparent K_m of the D214E protein for ACV was approximately threefold higher than that of the wild type, while the apparent k_{cat} was ~ 35-fold lower. Despite the potential difficulties in interpretation of these parameters [12], particularly in the case for mutant enzymes with low activity, the apparent k_{cat}/K_m values of the wild type and mutant enzymes presumably reflect their relative catalytic efficiency.

Table 3 lists the relative specific activities of the *S. jumonjinensis* IPNS compositional mutant proteins made in this study — shown in bold. For comparison, the table includes data from site-directed mutagenesis studies of other related dioxygenases, IPNS of *C. acremonium*, several ACC oxidases from different plant sources, flavanone 3β-hydroxylase of *Petunia hybrida* and human prolyl 4-hydroxylase. We discuss first the effects on activity of modifying the aspartic acid ligand and then those of modifying the two histidine ligands.

3.2. Aspartic acid active site mutants

Inspection of Table 3 shows that substitution of glutamic acid for aspartic acid in each of the above dioxygenases resulted in enzymes with partial activities that varied from $\sim 0.2\%$ for tomato ACC oxidase to $\sim 1\%$ for *S. jumonjinensis* IPNS and kiwi fruit ACC oxidase, and $\sim 15\%$ for human prolyl 4-hydroxylase. Replacement of the aspartic acid by asparagine in the petunia flavanone 3β-hydroxylase was reported to give an enzyme with $\sim 0.4\%$ residual activity, however, no detectable activity was found for this change for tomato ACC oxidase or human prolyl 4-hydroxylase. Although it was considered probable that replacement of aspartic acid with glutamic acid in the active site would not dramatically alter

Table 2
Kinetic parameters of the *S. jumonjinensis* wild type and the Asp214Glu mutant IPNS proteins

Enzyme	Relative specific activity	K_m (mM)[a]	k_{cat} (min^{-1})[a]	k_{cat}/K_m ($mM^{-1}min^{-1}$)
Wild type	1.0	0.9	12.0	13.5
Asp214Glu	0.01	2.57	0.33	0.12

[a] Results reported are the averages based on three measurements.

Table 3
Relative specific activities of active site mutants of IPNS and related oxidases

Modification of enzymes	Relative activity[a]						
	IPNS *S. jum.*[b]	IPNS *C. acr.*[b]	F3H Petunia[b]	ACCO Tomato[b]	ACCO Apple[b]	ACCO Kiwi[b]	P4H Human[b]
His212:							
Asn	nd				nd		
Asp	nd			nd			
Gln	nd		0.15	nd		1.0	
Ala	nd				nd		
Glu				0.2			nd
Leu		nd					
Phe					nd		
Asp214:							
Glu	1.0			0.2		1.0	15
His	nd				nd		
Cys	nd						
Asn			0.4	nd			nd
Ala	nd				nd		nd
Leu		nd					
His268:							
Asn	nd				nd		
Asp	nd			nd			
Gln	nd		nd	nd			
Ala	nd				nd		
Arg							nd
Leu		nd					
Phe					nd		

[a]Activity of purified modified enzymes expressed as percent of wild type; nd denotes no detectable activity ($< 0.2\%$); modified *Streptomyces jumonjinensis* IPNS proteins studied in this work indicated by bold letters.
[b]Other data from: *Streptomyces jumonjinensis* IPNS [7]; *Cephalosporium acremonium* IPNS [8,9]; petunia flavanone 3β-hydroxylase, F3H [13]; tomato fruit [12]; apple fruit [10]; and kiwi fruit [11] ACC oxidases, ACCO; human prolyl 4-hydroxylase, P4H [14].

the iron(II) coordination chemistry, the effects of replacing the aspartic acid with other residues such as histidine (which creates three histidines in the active site) or cysteine, both of which contain a functional group — the nitrogen atoms of the imidazole ring and the sulfur atom of the sulfhydryl group, respectively — with the potential to bind iron, and which can occur as ligands in the active site of IPNS and other ferrous iron proteins, were not obvious. Molecular modeling studies described below reveal that the positions of these atoms in the modified enzymes are not favorable for iron binding. The fact that only glutamic acid is able to substitute for aspartic acid in the above group of ferrous iron dioxygenases with partial retention of activity indicates that both the charge and side chain length of the aspartic acid ligand are important for normal activity.

3.3. Histidine active site mutants

Examination of Table 3 shows the effect on activity of individually replacing the two histidine ligands in different dioxygenases with other iron binding amino acids. Thus, substitution of glutamine for His212 in *S. jumonjinensis* IPNS and for the corresponding histidine in tomato ACC oxidase produced in both cases inactive enzymes, whereas the same modification in the petunia

flavanone 3β-hydroxylase and the kiwi ACC oxidase resulted in enzymes with ~0.15% and ~1% activity, respectively. Also, substitution of that histidine with glutamic acid in the tomato ACC oxidase resulted in an enzyme with ~0.2% activity while in the human prolyl 4-hydroxylase it produced an inactive enzyme. In contrast, replacement of His212 in *S. jumonjinensis* IPNS with asparagine and aspartic acid, and the corresponding histidine in the ACC oxidases of tomato and apple with aspartic acid and asparagine, respectively, created in each case inactive enzymes. Similarly, in those enzymes where the second histidine ligand, corresponding to His268 in *S. jumonjinensis,* was changed to an asparagine, aspartic acid or to a glutamine, the modified enzymes in all cases were completely inactive.

3.4. Activity of spatial IPNS mutant proteins

Two IPNS double mutant proteins were constructed — H212D, D214H and D214H, H268D — in which the arrangement of the two histidines and the aspartic acid in the wild type active site were changed to create a spatially different iron coordination center. Mutants of this type have not previously been described. Unexpectedly, both spatial mutants had no detectable activity (less than 0.2% of wild type), indicating that the activity of the native *S. jumonjinensis* IPNS depends on a unique spatial arrangement of the three protein ligands in the ferrous iron active site. The crystal structure of the *S. jumonjinensis* IPNS (see below) shows this arrangement to be identical to that reported for the *A. nidulans* IPNS [3]. In the latter case, the structure of the IPNS-Fe(II)-ACV substrate complex [4] reveals that the carboxylate ligand (Asp216) is positioned *trans* to the site where the dioxygen analog nitric oxide binds, and a similar arrangement of iron ligands occurs in the active site of 2,3-dihydroxybiphenyl dioxygenase [24]. In the deacetoxycephalosporin synthase-Fe(II) 2-oxoglutarate complex, the corresponding carboxylate ligand (Asp185) binds *trans* to the 2-carbonyl group of the cofactor, which is the equivalent position to the proposed binding site of dioxygen in IPNS [25]. This point is discussed further below.

3.5. Molecular modeling of compositional and spatial IPNS mutant proteins

The refined coordinates of the *S. jumonjinensis* apo-IPNS and the IPNS-Fe(II)-ACG (glycine) substrate inhibitor complex (the crystal structures will be described elsewhere) were used to model the effects on iron coordination of replacing the aspartic acid at position 214 with glutamic acid, cysteine and histidine, and of exchanging that aspartic acid with either His212 or His268. Comparison of the structures of the apo-IPNS and the IPNS inhibitor complex shows that the two structures are very similar, indicating that the IPNS structure does not change significantly upon binding of iron and ACG. It was assumed, therefore, in the molecular modeling analysis that the geometry of the protein main chain in the native protein is unchanged in the mutant proteins. Molecular modeling of the D214E protein showed that the longer side chain of the glutamic acid, compared to aspartic acid, causes the iron atom to be displaced. The most favorable arrangement of the protein ligands for coordination of the iron atom in the active site was found to be that in which the iron atom is shifted by ~1.4 Å from its native position — in the direction away from the D214 C_α atom (Fig. 1a). This movement is accompanied by rotations for His212 and His268 of 15° and 56° about χ_1, and 0° and 160° about χ_2, respectively. A similar analysis of the D214H and D214C mutant proteins showed that the histidine N^ε imidazole and the cysteine sulfhydryl atoms, respectively, are positioned too far from the iron atom to effectively contribute to its binding (data not shown). Molecular modeling of the H212D, D214H and the D214H, H268D spatial double mutant proteins, in which His212 and His268 are placed in *trans* to the putative binding site for dioxygen, showed that the relative positions and geometry of the aspartic acid carboxylate oxygen and the histidine imidazole N^ε liganding atoms are significantly changed compared to the wild type and are unfavorable for metal binding. In part this reflects the quite different distances separating the histidine (~6.2 Å) and the aspartic acid (~4.8 Å) C_α atoms from the iron atom in

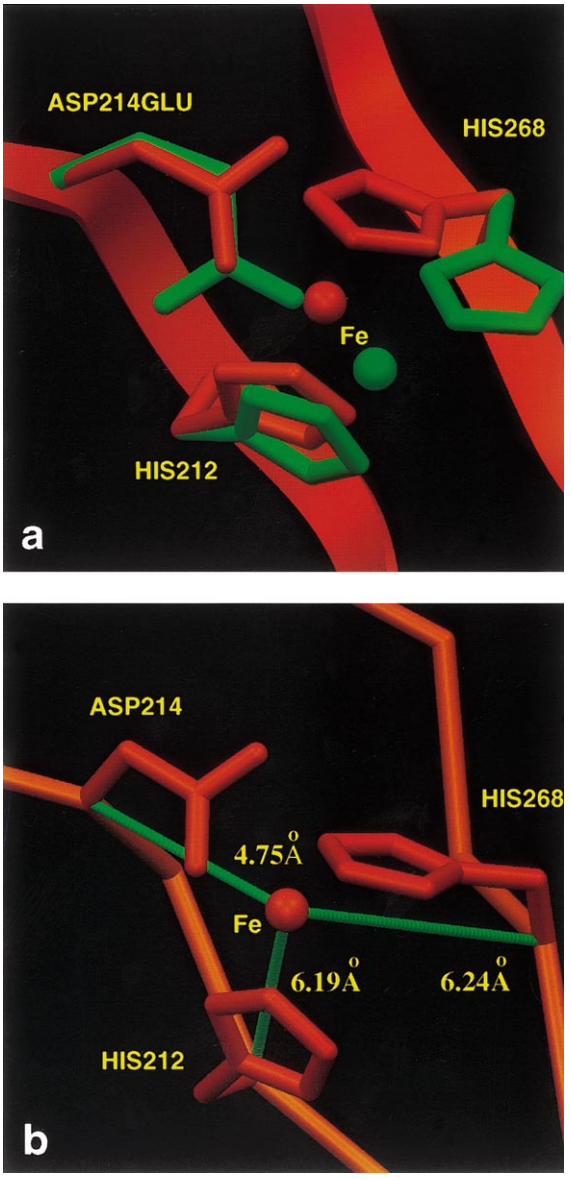

Fig. 1. (a) Superposition of the iron–protein ligands in the active site of native *S. jumonjinensis* IPNS (orange) with the corresponding ligands of the mutant Asp214Glu IPNS (green). (b) Non-symmetrical disposition of the Cα atoms of the His212, Asp214 and His268 iron ligands in the active site of native *S. jumonjinensis* IPNS.

the wild type protein (Fig. 1b). According to this analysis, two of the three endogenous iron ligands in the spatial mutants should be disrupted.

3.6. Iron binding

To evaluate whether the IPNS active site mutant proteins are affected in their ability to bind iron, the amount of iron bound by the wild type and selected mutant proteins was determined. The data reported in Table 4 have been corrected for extraneous iron binding to IPNS, determined as the amount of iron bound to a mutant IPNS protein in which all three active site protein ligands were exchanged with alanines. This protein is entirely lacking in activity as are each of the single alanine-substituted proteins [7]. In the conditions of our experiments, wild type IPNS bound ~0.75 iron atoms/protein molecule. The weakly active D214E enzyme bound less iron, ~0.31 iron atoms/protein molecule. We presume, on the basis of the molecular modeling analysis, that the iron present in its active site is displaced and bound in a way that precludes it from effectively promoting catalysis as compared with the wild type. In contrast the histidine mutant, D214H, and the D214C cysteine mutant, both of which are totally devoid of activity, bound almost the same amount of iron as the wild type enzyme. Thus, the presence in each of these mutant IPNSs of two of the native protein ligands, His212 and His268, is sufficient for effective metal binding. Taken together, these observations suggest that the aspartic acid ligand may have two functions,

Table 4
Iron binding by wild type and active site mutants of IPNS

Enzyme	Iron bound/enzyme[a]
Wild type	0.75
Asp214Glu	0.31
Asp214His	0.65
Asp214Cys	0.56
Asp214His, His268Asp	0.09

[a] Iron bound is expressed as equivalents of ferrous iron per mole enzyme and was calculated after subtraction of non-specific binding; the latter was determined as the amount of iron bound, in the same experimental conditions, by a mutant enzyme in which all three active site protein ligands had been replaced with alanines and was found to be 0.49 eq./mol enzyme. Results reported are the averages based on two to three experiments.

to bind iron and to participate in some other step in the catalytic cycle. Measurement of iron binding in the D214H, H268D spatial double mutant showed that the amount of iron bound was almost the same as the level of extraneously bound iron. This result is consistent with the molecular modeling studies described above which predict that in this mutant two of the protein ligands are abolished. Because of the uncertainty in the estimates of the iron bound at the active site, due to the existence of additional iron binding site(s), the interpretation of the results given in Table 4 should be viewed with some caution. In this respect it is noteworthy that the crystal structure of the *A. nidulans* IPNS complexed with manganese reveals two binding sites for manganese, one at the active site and one near the surface attached to glutamine and histidine residues [3].

4. Conclusions

The experiments reported here were designed to help better define the role of the endogenous IPNS ferrous iron ligands in catalysis. The crystal structures of the *A. nidulans* [3,4] and the *S. jumonjinenesis* IPNS (unpublished results), deacetoxycephalosporin C synthase [25,26], and several other non-heme ferrous iron oxygenases, all of which possess the 2-His-1-carboxylate triad, including tyrosine hydroxylase [27], 2,3-dihydroxybiphenyl 1,2 dioxygenase [24], soybean lipoxygenase [28] and iron superoxide dismutase [29], indicate that a primary role for the protein ligands is to anchor the iron atom in the active site while making available additional sites for binding of substrate, cofactors and dioxygen [6]. The results described here for IPNS compositional mutants, in which the active site protein ligands were replaced with residues possessing a functional group potentially able to bind iron, and for IPNS spatial mutants in which the protein ligands were cyclically rearranged in the active site, are consistent with their having a role in iron binding. Furthermore, they raise the possibility that the protein ligands, in particular the aspartic acid ligand, may also play an additional role in the catalytic cycle. This is suggested by the findings that replacement of the *S. jumonjinensis* Asp214 ligand with either histidine or cysteine resulted in completely defective enzymes but did not appreciably affect their ability to bind iron; on the other hand substitution of that aspartic acid with glutamic acid created an enzyme with a low, but significant, activity while substantially decreasing iron binding. These results demonstrate that the charge of the carboxylate ligand, the length of its side chain and its spatial position in the IPNS active site are all critical for function.

Elsewhere, it has been proposed that binding of substrate to IPNS and related oxygenases primes the iron center for binding of O_2 by modulating the redox potential of the iron center, and evidence in support of this has come from crystallographic and spectroscopic studies of several of the above cited enzymes (see [6]). In IPNS the charged aspartate carboxylate group is positioned *trans* to the presumed O_2 binding site and this may facilitate formation of an $Fe-O_2$ adduct which is thought to be a necessary step in the catalytic cycle [30]. Thus, not only the nature but also the precise spatial organization of the functional groups in the metal coordination center may be crucial for this to occur. It will be of interest therefore to analyze the ability of the two classes of IPNS mutant enzymes discussed in this work for their ability to bind ACV and the substrate analog ACG. One approach will be to investigate the metal catalyzed oxidative modification of the complexes as recently described for ACC oxidase [12]; another will be to employ spectroscopic means to measure the interaction of modified IPNSs with tripeptides.

Acknowledgements

We wish to thank Jim Remington for providing data for determining the crystal structure of the *S. jumonjinensis* IPNS, and for support from the Israel Science Foundation, Grant No. 488.97 and the Constantiner Institute for Molecular Genetics at Tel Aviv University.

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