FEBS Letters 405 (1997) 172–174 FEBS 18325

The glutamine ligand in the ferrous iron active site of isopenicillin N synthase of *Streptomyces jumonjinensis* is not essential for catalysis

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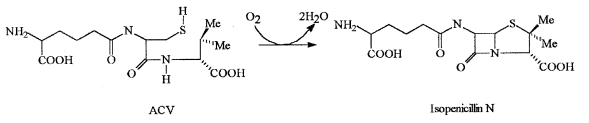
Received 16 January 1997; revised version received 5 February 1997

Abstract **Isopenicillin N synthase (IPNS) is a non-heme ferrous iron dependent dioxygenase that catalyses the ring closure of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N. We previously used site-directed mutagenesis to identify in the IPNS of *Streptomyces jumonjinensis* two histidines and one aspartic acid that are essential for activity. The recent crystal structure of the IPNS of *Aspergillus nidulans* establishes that these amino acids are iron ligands and reveals that the fourth ligand is the penultimate glutamine. The two histidines and one aspartic acid are conserved in several classes of non-heme ferrous iron dioxygenases, whereas the glutamine is present only in IPNSs. In this paper we show that the penultimate glutamine in *S. jumonjinensis* IPNS Gln-328 is not essential for catalysis. In contrast, Gln-230 which is highly conserved among the above dioxygenases and is proximal to the active site is crucial for activity.**

Key words: Isopenicillin N synthase; Nonheme iron dioxygenase; Protein ligand; Mutagenesis

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 [1,2]. In a unique reaction, IPNS catalyses the ring closure of δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) to form isopenicillin N during which four hydrogen atoms are transferred from the tripeptide substrate to completely reduce dioxygen to water [3,4].



Studies on the mechanism of action of IPNS have, in the last few years, focused mainly on the structure of the ferrous iron active site and its role in catalysis. In particular, spectroscopic studies have shown that the coordination environment of the iron atom in the holo-enzyme contains three histidines and one aspartic acid endogenous ligands, as well as sites for ACV, dioxygen and solvent [5–10]. According to this model, one of the protein ligands in the IPNS-ACV complex, presumably the axial histidine, is displaced on binding of oxygen. These findings have largely been substantiated in the recently reported crystal structure of the manganese form of *Aspergillus nidulans* IPNS [11]. The 3D structure reveals two histidine ligands, rather than three, and one aspartic acid ligand and that unexpectedly the fourth endogenous metal ligand is the penultimate glutamine.

In previous studies, we have used site-directed mutagenesis to identify which of the conserved histidines and aspartic acids present in microbial IPNSs are necessary for function [12]. We demonstrated that the IPNS of *Streptomyces jumonjinensis* contains two histidines, His-212 and His-268, and one aspartic acid, Asp-214, that are essential for activity. Moreover, these amino acid residues correspond precisely to the protein ligands seen in the crystal structure. We further showed by sequence analysis that the two histidines and the one aspartic acid are conserved in more than 50 enzymes belonging to several classes of non-heme ferrous iron dependent dioxygenases, including IPNS, 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO) and related 2-oxoglutarate dependent oxygenases, whereas the penultimate glutamine, Gln-328 in *S. jumonjinensis*, occurs only in IPNS. A second glutamine, Gln-230, that appears to be proximal to the active site, and might therefore be a potential ligand, is conserved in virtually all of the above non-heme iron dioxygenases. The purpose of this work is to determine by genetic and biochemical analyses the importance of Gln-230 and Gln-328 in IPNS for enzyme activity.

2. Materials and methods

Plasmid pOL18 containing the *S. jumonjinensis* IPNS structural gene under the control of a phage T7 promoter was used for the construction and expression in *E. coli* of mutant IPNS genes, as previously reported [13].

2.1. Synthetic oligodeoxyribonucleotides

5′-caagaacggcGCCacctgagcc and **5′-cagaacctcGCGgtcgagacg** were used as mutagenic primers for creating the Gln-328 and Gln-230 alanine substitutions, respectively. The altered nucleotides, compared to wild type, are shown in capital letters; in the Gln-328 substitution an *Nar*I restriction site is created and is shown underlined. Site-directed mutagenesis and DNA manipulations were performed as previously described [12]. The presence of mutations was verified by DNA sequencing. Fig. 1 shows the relevant DNA region of the IPNS gene containing the altered Gln-328 codon and the corresponding region of the wild-type gene.

Purification of wild-type and mutant recombinant IPNS proteins, enzymatic assays and determination of kinetic parameters were carried out as previously described [12,14,15].

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Abbreviations: ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine; IPNS, isopenicillin N synthase; ACCO, 1-aminocyclopropane-1-carboxylic acid oxidase; SDS, sodium dodecyl sulphate.

PII S0014-5793(97)00180-4

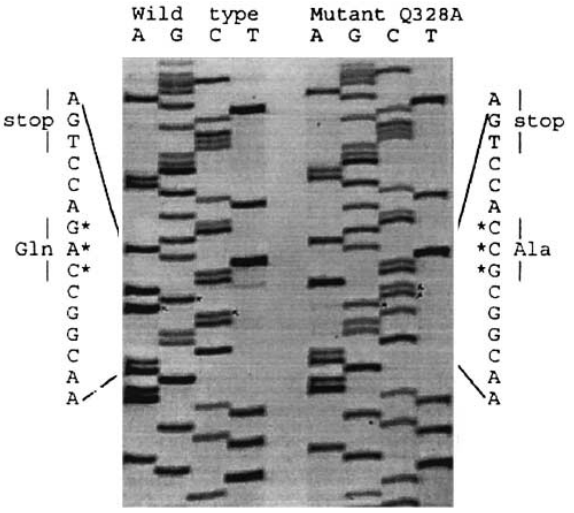

Fig. 1. Nucleotide sequence of the region containing the penultimate glutamine codon, CAG, in the wild-type IPNS of *S. jumonjinensis* and the alanine-substituted codon, GCC, in the Gln-328 mutant IPNS.

3. Results

S. jumonjinensis IPNS contains 11 glutamines, three of which are fully conserved among the 10 known IPNSs including the penultimate Gln-328. Site-directed mutagenesis was used to exchange the latter, as well as the conserved Gln-230, to alanine.

3.1. Expression of mutant IPNS genes

Fig. 2 shows the effect of the substitutions on synthesis of the recombinant proteins in *E. coli*. Both mutant proteins were made to about the same extent and possessed essentially the same mobility as judged by SDS-gel electrophoresis of the solubilized IPNS preparations. For comparison, the Gln-328 and Gln-230 alanine substituted mutant proteins are shown together with the wild-type protein as well as the previously reported His-212, Asp-214 and His-268 alanine substituted proteins.

3.2. Biochemical analysis of mutant IPNS enzymes

Wild-type and mutant proteins made with the T7 expression system were found to be almost entirely insoluble. Highly purified, solubilized, IPNS was recovered after centrifugation of cell sonicates, denaturation in urea and refolding as previously described [13]. The relative specific activities of the Gln-328 and Gln-230 mutant enzymes and that of wild-type IPNS prepared in this way are reported in Table 1. The Gln-328 mutant enzyme possesses appreciable activity, its specific activity is approx. 20% that of wild type. In marked contrast, the Gln-230 mutant enzyme had no detectable activity.

Kinetic parameter determinations show that the K_m of the Gln-328 mutant is not significantly different from that of wild type, whereas the k_{cat} is some 5–6-fold less. These constants could not be measured for the Gln-230 inactive enzyme.

4. Discussion

Spectroscopic studies of IPNS and its substrate complex support a model in which the metal atom in the active site is attached to four protein ligands, presumed to be three histidines and one aspartic acid (reviewed in [16]). In addition, the studies indicate that one of the protein ligands is displaced on binding of oxygen to the enzyme substrate complex, plausibly the axial histidine ligand [8,10]. We previously showed using site-directed mutagenesis that His-212, Asp-214 and His-268 in *S. jumonjinensis* IPNS are essential for enzymatic activity. Furthermore, sequence analysis of more than 50 isopenicillin N synthase, 1-aminocyclopropane-1-carboxylic acid oxidase and related 2-oxoglutarate dependent oxygenases revealed that all possess precisely these three residues [12]. These results led us to propose that the above residues constitute three of the four postulated IPNS protein ligands, and we speculated that the remaining ligand could not be a histidine or aspartic acid.

Compelling evidence for the above view has come from the recently derived 3D structure of the *A. nidulans* IPNS [11]. Crystals of IPNS were prepared with manganese replacing iron at the active site. The structure of the active site reveals four protein ligands bound to the metal atom, three of which correspond exactly to those identified by the genetic and sequence analysis of the ferrous active site of the *S. jumonjinensis* IPNS. Surprisingly, the fourth ligand was found to be glutamine (Gln-330, corresponding to Gln-328 in *S. jumonjinensis* IPNS), the penultimate residue in the molecule.

In this work we used site-directed mutagenesis to determine whether the penultimate glutamine residue in the *S. jumonjinensis* IPNS is necessary for enzymatic activity. Our results demonstrate that Gln-328 is not essential for catalysis since the alanine substituted enzyme possesses significant activity. In view of this finding we propose that the essential two histidines and one aspartic acid ligands are permanent active-site ligands and are bound to iron throughout the reaction cycle, whereas the penultimate glutamine, rather than a histidine, is the replaceable ligand predicted by spectroscopic studies. This idea is consistent with the 3D structure of the octahedral coordination environment of the metal center, which shows that all three essential protein ligands and ACV, bound through its cysteinyl thiolate, lie in the same equatorial plane, and that consequently the remaining fourth ligand in the en-

Table 1
Kinetic parameters of *S. jumonjinensis* IPNS Gln-230→Ala and Gln-328→Ala mutants

IPNS mutant	Relative specific activity	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild type	1.0	0.68	14.0	17.3
Gln-230	<0.003	n.m.	n.m.	n.m.
Gln-328	0.16	0.58	2.0	3.5

Recombinant proteins were made in *E. coli* employing a T7-based expression system [13]. Specific activities of solubilized IPNS preparations were determined by bioassay and HPLC and kinetic parameters were determined by HPLC assay as previously described [14,15]. Values reported are the average numbers based on more than five determinations from two independently made protein preparations; measurements varied about the mean values by no more than 25%; n.m., not measurable.

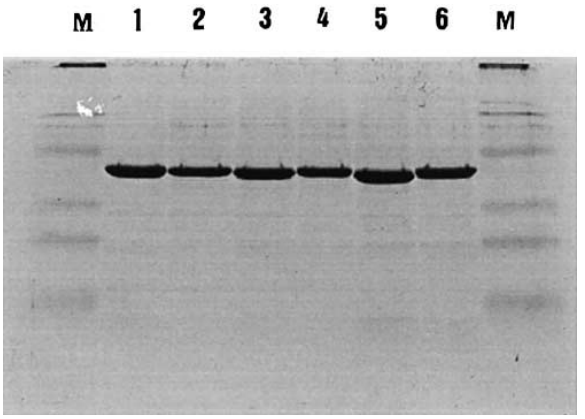

Fig. 2. SDS-polyacrylamide gel electrophoresis of *S. jumonjinensis* wild-type and mutant IPNS proteins. Recombinant proteins were prepared as previously described [13]; lanes 1–6 in ascending order, wild-type, His-212, Asp-214, His-268, Gln-230 and Gln-328 mutant IPNS proteins. M, molecular mass standards: 20, 24, 29, 36, 43 and 66 kDa.

zyme-substrate complex occupies an axial position and is the one displaced on oxygen binding.

Several questions arise concerning the nature and role of the displaceable iron ligand in IPNS. Glutamine has not previously been definitively reported as a ligand in non-heme iron proteins, whilst the related asparagine has been implicated as a non-heme iron ligand in one case only, that of soybean lipoxygenase [17]. The finding that the penultimate glutamine in the IPNS of *A. nidulans* is an iron ligand is especially intriguing since this residue is not conserved in the other non-heme ferrous iron dioxygenases referred to above. In this respect it has been pointed out by the authors of the 3D structure that differences in the coordination chemistry between IPNS and other members may reflect differences in reaction mechanism and substrate specificity and, in particular, the fact that IPNS does not use 2-oxoglutarate as a cofactor [11]. However, the finding that the ACCO class of iron dioxygenases does not require 2-oxoglutarate and lacks glutamine in the penultimate position in the molecule appears to rule out the latter possibility. The same authors also mention that the crystal structure of the *A. nidulans* IPNS reveals that the carboxy terminus of the polypeptide chain exhibits a strained geometry and that the glutamine metal (manganese) ligand has an atypical conformation [11]. In the light of these considerations we have sought through sequence analysis to identify other potential iron ligands in IPNS. Two candidates are His-48, which is conserved in the great majority of the above non-heme iron dioxygenases, and Gln-230 which is likewise almost entirely conserved and which appears from the published pictures of the 3D structure to be proximal to the metal center. We have previously shown that substitution of His-48 with alanine results in an enzyme with about 20% the specific activity of the wild type [12], similar to that found with the Gln-328 alanine exchange reported here. On the other hand, we show here that substitution of Gln-230 with alanine results in a completely inactive enzyme. Possibly, Gln-230, which is proximal to the active site, is crucial for catalysis because it functions as a weak extended non-heme ligand analogous to the asparagine ligand in soybean lipoxygenase [18]. These findings we believe will stimulate the need for further investigations on the IPNS active-site ligands, especially in determining their role in iron binding and in substrate interactions. The mutants described here and previously should be invaluable for these studies.

Acknowledgements: We thank the Israel Binational Science Foundation for a grant supporting this work, Grant No: 93-00097. We thank Dr. C. Schofield for comments.

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