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Mutagenesis Studies on the Iron Binding Ligands of Clavaminic Acid Synthase

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Mutagenesis studies on conserved histidine residues identified as possible metal binding ligands in clavaminic acid synthase isozyme 2 were consistent with His-145 and His-280 acting as iron ligands, in support of crystallographic and previous mutagenesis studies. Mutagenesis of the four cysteines and a glutamine residue, conserved in both clavaminic acid synthase isozymes 1 and 2, demonstrated that none of these residues is essential for activity.

Key Words: **clavaminic acid synthase; nonheme iron; 2-oxoglutarate; oxygenase; site-directed mutagenesis.**

Clavaminic acid synthase (CAS) catalyzes three steps in the bacterial biosynthesis of the class A β-lactamase inhibitor clavulanic acid. CAS is a member of a large family of nonheme iron (II)-dependent oxygenases, all but two of which use 2-oxoglutarate (2OG) as a cosubstrate (1, 2). These enzymes catalyze a variety of different metabolic reactions and may be as ubiquitous as their heme-dependent counterparts. CAS is remarkable in that it catalyses three different types of oxidative reaction during clavam biosynthesis. One of its reactions, hydroxylation, is separated from the other two, cyclization and desaturation, by the action of an amidinohydrolase (3–5).

Abbreviations used: 2OG, 2-oxoglutarate; ACC, 1-amino-1-carboxylate-cyclopropane; CAS, clavaminic acid synthase; CD, circular dichroism; DAOCS, deacetoxycephalosporin C synthase; DTT, dithiothreitol, ESI–MS, electrospray ionization–mass spectrometry; FPLC, fast protein liquid chromatography; IPNS, isopenicillin N synthase; IPTG, isopropyl-β-D-thiogalactoside; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; PEI, polyethyleneimine; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid; TSP, (2,2′,3,3′)-[$^2H_4$]-(trimethylsilyl)propanoic acid.

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In *Streptomyces clavuligerus* there are two iso-forms of CAS (CAS1 and CAS2) which are 87% identical at the protein level (6). Recently, crystal structures of CAS1 complexed to iron, 2OG and prime substrates were reported (7). Crystal structures have also been reported for another 2OG oxygenase deacetoxycephalosporin C synthase (DAOCS) (8, 9) and the structurally related enzyme, isopenicillin N synthase (IPNS) (10), which does not use 2OG as cosubstrate. The CAS structure revealed that it contained a similar jelly-roll core of β-strands to those found in DAOCS and IPNS despite little sequence similarity being apparent in the absence of the crystal structures (6, 7).

All nonheme oxygenases for which structures have been reported appear to contain a facial 2-His-1-carboxylate triad of residues, which are responsible for iron ligation by the protein (11). CAS appears to be unusual among the 2OG oxygenases in that the carboxylate is provided by a glutamyl rather than an aspartyl side chain (Fig. 1). Prior to the determination of the crystal structure it was speculated that a conserved HXE motif in CAS provided two of the likely three protein ligands for iron (4).

Using site-directed mutagenesis, a series of histidine residues conserved in both CAS1 and CAS2 were converted to leucines to test this speculation. The results are supportive of the crystallographic studies (7) and a recently reported study in which the likely iron binding histidines of CAS2 were converted to glutamine residues (12).

MATERIALS AND METHODS

Materials. All chemicals were purchased from the Sigma–Aldrich Chemical Co. or E. Merck unless otherwise stated, and were of at least analytical grade. Oligonucleotides were synthesized by V. Cooper (The Dyson Perrins Laboratory, Oxford University) using Applied Biosystem DNA synthesizers (Models 380B and 394). The following supplied equipment and chemicals: FPLC systems and columns (Amersham Pharmacia Biotech); HPLC columns (Phenomenex); 1-kb markers (MBI); enzymes for molecular biology and com-

0006-291X/00 $35.00

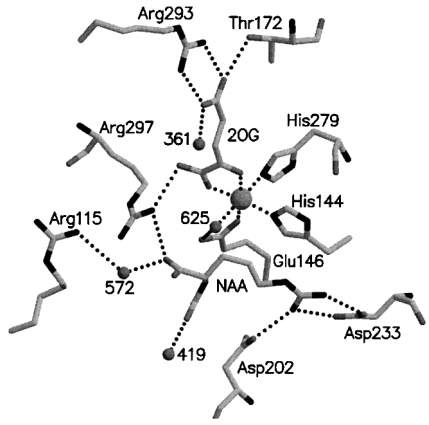

FIG. 1. View of the CAS active site derived from the crystal structure of CAS1 complexed to iron(II) and 2OG in the presence of *N*-α-acetyl-L-arginine (NAA). The numbering of the equivalent residues in CAS2 is plus one. Important residues for iron (central sphere) and substrate binding are shown. Numbered spheres are water molecules.

petent cells (NEB and Stratagene); Pro-Wizard plus DNA purification system (Promega).

Molecular biology protocols and site-directed mutagenesis. CAS2 encoding gene, cas2, was isolated from *S. clavuligerus* by a previously published method (13) using PCR and cloned into the plasmid vector, pET11a (Novagen), using *Nde*I and BamHI restriction enzymes. DNA concentrations were determined at $A_{260\,nm}$ according to standard protocols (14). Plasmid DNA was purified using the Pro-Wizard miniprep system. Oligonucleotide primers (Fig. 2) were deprotected in ammonia and precipitated according to standard instructions (14). Site-directed mutagenesis was performed using the Stratagene QuikChange system. DNA samples were analyzed by gel electrophoresis using 0.8% (w/v) agarose gels. The resulting plasmids were transformed into competent *Escherichia coli* XL1 Blue and BL21 (DE3) cell strains and grown according to reported instructions (13). The sequences of the mutants were confirmed by fluorescent automated DNA sequencing (Department of Biochemistry, Oxford University), and protein expression confirmed by growth of a single colony. Samples were analyzed by SDS–PAGE.

Enzyme expression, purification, and analysis. Recombinant *E. coli* BL21 (DE3) cells were cultured and crude extracts prepared according to standard protocols (13). All operations were conducted at 4°C. Protein concentrations were estimated by the Bradford method (15) using BSA as a standard at $A_{595\,nm}$. Wet cell pellets (ca. 15 g) were resuspended to 3× (w/v) of lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM DTT, 2 mM EDTA, 100 mM NaCl, 0.2 mg/mL lysozyme, 0.5 mM PMSF) and sonicated using an Ultra-Sonics W-380 sonicator. Extracts were first centrifuged and then DNA precipitated using PEI as reported (13). Clarified soluble extracts were purified using a Q-Sepharose 60/100 column equilibrated in buffer A (50 mM Tris–HCl, pH 8.0, 1 mM DTT, 1 mM EDTA) and eluted with a 0–500 mM NaCl gradient over 500 mL, made with buffer A. Fractions were analyzed by SDS–PAGE. Fractions containing CAS were pooled and concentrated to <8 mL using an Amicon stirred cell fitted with a PM-10 membrane. Samples were loaded onto a Superdex-75 gel-filtration column equilibrated with buffer A supplemented with 100 mM NaCl. Protein was eluted with the same buffer at 2 mL/min. Fractions were analyzed by SDS–PAGE, pooled and protein concentrated to ca. 10 mg/mL. Purified protein (>90% purity) was characterized by CD analyses (Jasco JA710) over a 190- to 250-nm range and ESI–MS analyses. H145E and H145Q mutants were purified using a modified protocol, employing a hydrophobic interaction chromatography step inserted after the initial anion-exchange purification due to initial low expression levels. Samples in 1.6 M ammonium sulfate were loaded onto a SOURCE-ISO column (2.6 cm × 10 cm, 53 mL) equilibrated in buffer A containing 1.6 M ammonium sulfate. The column was washed with the same buffer at 5 ml/min, and eluted with a 1.6–0.8 M $(NH_4)_2SO_4$ gradient over 240 mL. Fractions were analyzed by SDS–PAGE following initial TCA precipitation. The required fractions were pooled and concentrated before final purification by gel-filtration chromatography as above.

Hydroxylation assay. HPLC-based assays were carried out in triplicate (at least) as described, employing *N*-α-acetyl-L-arginine as substrate analogue (16), except that DTT was omitted from the reaction (17). The filtered supernatant was injected onto a C_{18} Hypersil column (250 × 4.6 mm), eluting isocratically with Milli-Q water at 1.5 mL/min, monitored at 218 nm and 0.2 AUFS. The hydroxylated product was eluted with retention time of ca. 3.5 min and quantified using a standard curve of authentic hydroxylated material. ^{1}H NMR and ESI–MS analyses confirmed the identities of the HPLC purified products. For the ^{1}H NMR analyses of products, samples were dissolved in D_2O (referenced to TSP) and analyzed using a Bruker AMX 500 MHz spectrometer.

RESULTS AND DISCUSSION

The identities of the mutants in the cas2 gene were confirmed by DNA sequencing and by ESI-MS analyses of the purified proteins (Table 1). All mutants were expressed as soluble proteins and purified in a similar manner to the wild-type enzyme. The mutants were purified to >90% purity as judged by SDS–PAGE analyses, except for C8L, C200L, C201L, and Q154L mutants which were ca. 50% pure (Fig. 3). Circular dichroism analyses implied that the gross secondary con-

H109L	**5'** CGC CGG CTC GGT CTG **CTC** ACG GGG TAC CAG GAG **3'**
H122L	**5'** C TCG GGC ACG GTC TAC **CTC** GAC GTG TAC CCG TCG **3'**
H131L	**5'** CCG TCG CCC GGC GCG **CTC** TAC CTG TCC TCG GAG **3'**
H145L	**5'** G ACG CTG CTG GAG TTC **CTC** ACG GAG ATG GCG TAC **3'**
H145E	**5'** G ACG CTG CTG GAG TTC **GAG** ACG GAG ATG GCG TAC **3'**
H145Q	**5'** G ACG CTG CTG GAG TTC **CAG** ACG GAG ATG GCG TAC **3'**
H151L	**5'** C ACG GAG ATG GCG TAC **CTC** ATC CTC CAG CCG AAC **3'**
H167L	**5'** C TGC TCC CGC GCG GAC **CTC** GAG AAC CGG GCG GAG **3'**
H280L	**5'** C AAC TTC CGC ACC ACG **CTC** GCG CGG ACG CCG TTC **3'**
H297L	**5'** G AAG GAC CGC TGG CTG **CTC** CGC GTC TAC ATC CGC **3'**
C8L	**5'** C TCT CCG ATA GTT GAC **TTA** ACC CCG TAC CGC GAC **3'**
C162L	**5'** C TAC GTC ATG CTG GCC **TTA** TCC CGC GCG GAC CAC **3'**
C200L	**5'** GAC CGC AAG GTG CCC **TTA** TGC GTG GAC GTG GCC **3'**
C201L	**5'** CGC AAG GTG CCC TGC **TTA** GTG GAC GTG GCC TTC **3'**
Q154L	**5'** GCG TAC CAC ATC CTC **CTG** CCG AAC TAC GTC ATG **3'**

FIG. 2. Primers used in site-directed mutagenesis for generating CAS2 mutants. Primers were made in complementary pairs and are extended in the 5′ to 3′ direction during site-directed mutagenesis. Highlighted bold are the relevant mutant codons.

TABLE 1

Observed Masses for the CAS2 Wild-Type and Mutant Enzymes as Determined by ESI–MS

CAS2 types	Calculated MW (Da)	Observed MW (Da)
Wild-type	35,665	35,665 ± 2
H109L	35,641	35,642 ± 1
H122L	35,641	35,644 ± 4
H131L	35,641	35,640 ± 6
H145L	35,641	35,643 ± 4
H151L	35,641	35,640 ± 3
H167L	35,641	35,644 ± 2
H280L	35,641	35,640 ± 3
H297L	35,641	35,642 ± 3
C162L	35,675	35,675 ± 2

formations of the mutants were similar to the wild-type enzyme.

Initially, the histidines, conserved between the two CAS isozymes, were converted to leucine residues (H109L, H122L, H131L, H145L, H151L, H167L, H280L, and H297L). These results, carried out on the CAS2 isozyme, complement and reinforce those of Khaleeli *et al.* (12) who have converted the same histidines to glutamine residues (Table 2).

Significant activity (>15%) was maintained for all the histidine mutants except for the H122L, H145L, and H280L mutants where the level of activity was less than 5% that of the wild-type enzyme. The lack of activity observed for the H145L and H280L mutants reinforces their assignment from crystallographic studies as iron ligands at the CAS active site. The H145L mutant also did not convert 2OG to succinate above background levels as assayed by the ^{1}H NMR spectroscopic method (16). The H145E and H145Q mutants were also made and found to be inactive. The latter observation supports the results of Khaleeli *et al.* (12).

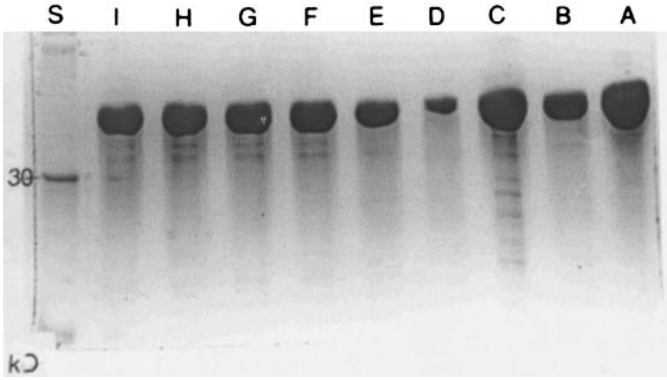

FIG. 3. An SDS–PAGE gel of wild-type CAS2 and histidine mutants [S, protein MW standard (30 kDa); I, H297L; H, H280L; G, H167L; F, H151L; E, H145L; D, H131L; C, H122L; B, H109L; A, wild-type CAS2].

TABLE 2

CAS2 Wild-Type and Mutant Activities as Determined by the HPLC Hydroxylation Assay

CAS2 types	S.A. (IU/mg)	Relative specific activity (%)
Wild-type	480	100
H109L	414	86
H122L	<5	<1
H131L	171	36
H145L	<5	<1
H145E	<5	<1
H145Q	<5	<1
H151L	90	19
H167L	414	86
H280L	33	3
H297L	329	69
C8L	200	42
C162L	482	100
C200L	200	42
C201L	190	40
Q154L	110	23

Note. Activities were determined using *N*-α-acetyl-L-arginine as a substrate analogue and according to instructions under Materials and Methods.

His-145 forms part of a conserved HXE iron-binding motif in CAS2. In the case of ACC oxidase, production of the analogous mutant (H177E) led to a modified ACC oxidase with a very low level of activity (18). However, it is not clear that this mutant ACC oxidase catalyzed the reaction in the same manner as the wild-type enzyme, since it can be mediated by nonenzymatic oxidizing conditions in contrast to the 'difficult' oxidation of inactivated carbon-hydrogen bonds as catalyzed by CAS.

The lack of activity for the H122L mutant (<5%) contrasts with the H122Q mutant which Khaleeli *et al.* (12) reported to be almost as active as the wild-type enzyme. His-122 is located on the first strand of the jelly-roll core of CAS2 (β-2 strand). The crystal structures of the CAS1 isozyme imply that His-122 of CAS2 is quite close to the active site. In the CAS1 structure, the C_{α}-carbon residue of His-121 is ca. 11.5 Å from the iron with its side chain projects toward the active site cavity and, in particular, the side chains of Tyr-299 and Arg-297. The latter is most probably involved in binding the substrate carboxylate and the former forms part of the β-lactam ring binding pocket. The difference in activity between the H122L and H122Q mutants may reflect a greater disruption of the active site by the introduction of the hydrophobic leucine residue. Although the H122L mutant displayed little activity during the hydroxylation assay with *N*-α-acetyl-L-arginine, it cannot be ruled out that it will catalyze the conversion of 2OG to succinate.

Reduced levels of activity were observed for the H109L, H131L, H151L, H167L, and H297L mutants. Qualitatively, the results follow the same trends as

those reported by Khaleeli *et al.* (12) for the analogous glutamine mutants. Without detailed kinetic analyses, quantitative rationalization of these results is difficult. The results will be briefly discussed in context of the crystal structures for the CAS1 isozyme (7). Except where stated the CAS2 numbering system is used.

Although the side chain of His-108 in CAS1 (analogous to His-109 of CAS2) is directed toward the interior of the protein, its C_α-carbon is located ca. 16 Å from the iron in the N-terminal alpha-helical region. Thus, it seems reasonable that a high level of CAS hydroxylation activity is maintained upon its mutation. The C_α-carbon of His-130 of CAS1 (His-131 of CAS2) is ca. 14 Å from the iron and is located on a loop which extends close to the active site linking the first and second strand of the jelly-roll structure. Its side chain is directed away from the active site, so it is probably not involved in substrate binding in the catalytically active enzyme–substrate complex, but may be involved in product release or the initial stages of substrate binding. The side chain of His-151 of CAS2 (C_α-carbon ca. 14 Å from iron, His-150 of CAS1) is separated from the active site by the side-chain of Met-148 and is proximal to Glu-147 (third residue of the 2-His-1-carboxylate triad). Thus, its mutation may indirectly disrupt the active site, explaining the reduced levels of activity observed. His-167 of CAS2 (C_α-carbon ca. 21 Å from iron, His-166 of CAS1) is located on a loop linking the third and fourth strands of the jelly-roll and is located on the exterior of the protein, consistent with the small reduction in activity observed for this mutant. His-297 (C_α-carbon ca. 12 Å from iron, His-296 of CAS1) is adjacent to Arg-298 (Arg-297 of CAS1) which is most probably involved in binding the substrate carboxylate group. The relatively high level activity (ca. 69%) observed for this mutant implies that any role His-297 plays in binding the substrate carboxylate is secondary to other residues such as Arg-298.

To aid in early crystallization trials, four cysteine residues (C8, C162, C200, and C201), conserved between CAS1 and CAS2, were also mutated to leucine residues. All four mutants displayed significant levels of activity. The C8L (C_α-carbon ca. 25 Å from iron, Cys-6 of CAS1), C200L (C_α-carbon ca. 16 Å from iron, Cys-199 of CAS1) and C201L (C_α-carbon ca. 15 Å from iron, Cys-200 of CAS1) mutants all displayed ca. 40% of the specific activity of the wild-type enzyme after partial purification (ca. 50% pure by SDS–PAGE analysis). It is reasonable that the highly purified mutants would possess higher specific activities. One of the mutants, C162L was purified to >90% purity as determined by SDS–PAGE analysis, was found to be at least as active as the wild-type enzyme. The side chain of Cys-162 (C_α-carbon ca. 14 Å from iron, Cys-161 of CAS1) points toward the binding site and the 5-carboxylate side chain of 2OG. It is possible that conversion of cysteine residues to less easily oxidized residues may be a way of obtaining a form of CAS more stable under the *in vitro* incubation conditions by avoiding the adventitious oxidation of thiols.

It is possible a glutamine side chain plays a role in the catalytic cycle of the nonheme oxygenase IPNS, which is related to CAS by primary sequence (19). Thus, a single glutamine residue, Gln-154 in CAS2, conserved in the CAS1 sequence via Gln-153, was mutated to leucine. The resultant protein demonstrated a reduced but significant level of activity demonstrating it is not essential for catalysis. The C_α-carbon of Gln-153 in CAS1 is ca. 12 Å from the iron. The modification of the analogous Gln-154 side chain in CAS2 may disrupt the active site, as the terminus of Gln-153 of CAS1 is only ca. 4 Å from the backbone carbonyl of Glu-146, one of three important iron-binding ligands in CAS.

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