Structural and Functional Studies of *Aspergillus clavatus* N⁵-Carboxyaminoimidazole Ribonucleotide Synthetase†,‡

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**Abstract:** N⁵-Carboxyaminoimidazole ribonucleotide synthetase (N⁵-CAIR synthetase), a key enzyme in microbial de novo purine biosynthesis, catalyzes the conversion of aminoimidazole ribonucleotide (AIR) to N⁵-CAIR. To date, this enzyme has been observed only in microorganisms, and thus, it represents an ideal target for antimicrobial drug development. Here we report the cloning, crystallization, and three-dimensional structural analysis of *Aspergillus clavatus* N⁵-CAIR synthetase solved in the presence of either Mg₂ATP or MgADP and AIR. These structures, determined to 2.1 and 2.0 Å, respectively, revealed that AIR binds in a pocket analogous to that observed for other ATP-grasp enzymes involved in purine metabolism. On the basis of these models, a site-directed mutagenesis study was subsequently conducted that focused on five amino acid residues located in the active site region of the enzyme. These investigations demonstrated that Asp 153 and Lys 353 play critical roles in catalysis without affecting substrate binding. All other mutations affected substrate binding and, in some instances, catalysis as well. Taken together, the structural and kinetic data presented here suggest a catalytic mechanism whereby Mg₂ATP and bicarbonate first react to form the unstable intermediate carboxyphosphate. This intermediate subsequently decarboxylates to CO₂ and inorganic phosphate, and the amino group of AIR, through general base assistance by Asp 153, attacks CO₂ to form N⁵-CAIR.

Arguably, one of the most important developments in the history of modern medicine has been the discovery of antibiotics. The golden age of antibacterial drug discovery began during the 1940s, and by the beginning of the 1970s, most of the major classes of antibiotics currently in clinical use had been discovered (1, 2). The rate of antimicrobial drug discovery has since slowed as evidenced by the fact that only two new classes of antibiotics, the lipopeptides and the oxazolidinones, have been marketed since the 1940s, and by the beginning of the 1970s, most of the major antibacterial agents are still in use.

By the end of 2009, it is estimated that one in three hospital patients is infected with bacterial pathogens resistant to existing antibiotics (3). Recently, studies have shown that approximately 50% of all Staphylococcus aureus infections are methicillin resistant, and strikingly, *S. aureus* resistance to most other existing antibiotics has been detected in clinical settings (1, 5, 6). These results foreshadow the day in which bacteria become resistant to all known antibiotics.

The increasing prevalence of antibiotic resistant infections has led to a recent, renewed interest in antibiotic drug development. For example, studies have examined enzymes such as pantothenate kinase (7), tRNA synthetase (8), and DNA ligase (9), or biosynthetic pathways such as those for the production of isoprenoids (10) and aromatic amino acid synthesis (11), as potential antibacterial drug targets. One unexplored pathway for antimicrobial drug design is de novo purine biosynthesis, which is markedly different in microorganisms and humans (12-16). As revealed in studies conducted in the 1980s, bacteria, yeast, and fungi synthesize the purine intermediate, inosine monophosphate (IMP), via 11 enzymatic steps, whereas in humans, only 10 steps are required. The additional step is catalyzed by the enzyme N⁵-carboxyaminoimidazole ribonucleotide synthetase, which converts 5-aminomimidazole ribonucleotide (AIR) to N⁵-carboxyaminoimidazole ribonucleotide (N⁵-CAIR) as indicated in Scheme 1 (12, 13, 16). This activity is not required for the synthesis of IMP in humans, and since humans have no homologues for this enzyme, N⁵-CAIR synthetase represents an attractive target for drug development. Indeed, we have discovered a series of small, druglike inhibitors of this enzyme and have shown that these agents inhibit bacterial growth on purine deficient media (17).

To further our drug discovery efforts and to enhance our understanding of N⁵-CAIR synthetase, we embarked on a series of structural and enzymological investigations. Recently, our laboratories reported the molecular structures of the Mg₂ATP and Mg/ADP-P₁ complexes of N⁵-CAIR synthetase from *Escherichia coli* (18). These two models provided us with a detailed view of the protein before and after ATP hydrolysis, and we were able to use this information to suggest a possible catalytic

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‡Abbreviations: AIR, 5-aminoimidazole ribonucleotide; AMPPNP, adenosine 5′-β,γ-imido)triphosphate; CAIR, 4-carboxy-5-aminoimidazole ribonucleotide; CHES, N-cyclohexyl-2-aminoethanesulfonic acid; MWC, molecular weight cutoff; Tris, tris(hydroxymethyl)-aminoethane.
mechanism for the enzyme. Unfortunately, numerous efforts aimed at obtaining the structure of the enzyme in the presence of AIR failed. Due to the mechanistic, structural, and medicinal importance of this information, we initiated a study of \( N^5 \)-CAIR synthetase from *Aspergillus clavatus*, a common soil fungus. As observed in other eukaryotic microorganisms, the *A. clavatus* \( N^5 \)-CAIR synthetase exists as a bifunctional protein with the next enzyme in the pathway, \( N^5 \)-CAIR mutase (16). For this investigation, we cloned only the gene encoding the enzyme via site-directed mutagenesis to probe their function within the active site of the enzyme. Taken together, these studies provide new, detailed information on those residues critical for substrate binding and for catalysis in the \( N^5 \)-CAIR synthetases.

**MATERIALS AND METHODS**

**Cloning, Expression, and Purification.** Genomic DNA from *A. clavatus* (ATCC 1007) was isolated by standard procedures. The fragment of the ADE2 gene encoding \( N^5 \)-CAIR synthetase (residues 1–383) was polymerase chain reaction-amplified using primers that introduced 5'-A-tailed and ligated into a pGEM-T (Promega) vector for screening and sequencing. Subsequently, two introns present in the gene encoding \( N^5 \)-CAIR synthetase were excised by mutagenesis. Purification of the enzyme was achieved via the hanging drop method against a sparse matrix screen developed at AIR. In addition, we changed five key residues in the AIR binding pocket (Glu 73, Tyr 152, Asp 153, Arg 155, and Lys 353) via site-directed mutagenesis.

**Structural Analysis of \( N^5 \)-CAIR Synthetase.** Crystallization conditions were initially surveyed by the hanging drop method of vapor diffusion with a sparse matrix screen developed in the laboratory, using the protein incubated with 10 mM ATP and 40 mM MgCl\(_2\). X-ray diffraction quality crystals were grown in 10 mM ATP, 40 mM MgCl\(_2\) solution containing 400 mM NaCl, 10 mM MgCl\(_2\), and 15% ethylene glycol.

Table 1: X-ray Data Collection Statistics

<table>
<thead>
<tr>
<th></th>
<th>enzyme complexed with Mg(_2)ATP</th>
<th>enzyme complexed with MgADP and AIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>space group</td>
<td>( P_2_1 )</td>
<td>( P_2_1 )</td>
</tr>
<tr>
<td>resolution limits (( \AA ))</td>
<td>30.0–2.1</td>
<td>30.0–2.0</td>
</tr>
<tr>
<td>no. of independent reflections</td>
<td>104,348 (127,400)</td>
<td>118,161 (157,172)</td>
</tr>
<tr>
<td>completeness (%)</td>
<td>93.6 (86.5)</td>
<td>91.9 (86.4)</td>
</tr>
<tr>
<td>redundancy</td>
<td>4.0 (1.8)</td>
<td>2.9 (1.5)</td>
</tr>
<tr>
<td>avg I/avg o(I)</td>
<td>9.6 (2.2)</td>
<td>7.3 (2.0)</td>
</tr>
<tr>
<td>( R_{sym} ) (%)</td>
<td>8.7 (30.9)</td>
<td>8.9 (28.6)</td>
</tr>
</tbody>
</table>

*\( R_{sym} = (\sum |I| - \sum |I|)/\sum |I| \times 100. \) Statistics for the highest-resolution bin.

![Scheme 1](image-url)
enzymes were determined using the ATP-coupled assay system as previously described (17). In a 1 mL cuvette, buffer [50 mM HEPES (pH 7.5), 20 mM KCl, and 6.0 mM MgCl₂], 0.2 mM NADH, 2.0 mM phosphoenolpyruvate, 1.0 mM NaHCO₃, 4 units of pyruvate kinase, and 17 units of lactate dehydrogenase were added. After the cuvette was equilibrated to 37 °C, N⁵-CAIR synthetase was added (the amount varied depending upon the mutant protein) followed by 1.1 mM ATP. Background levels of ATP hydrolysis were measured, and the reaction was initiated by the addition of AIR (concentration varied from 5 to 500 μM). NADH oxidation was monitored at 340 nm, and the concentration of AIR was determined at 250 nm. The concentration of AIR was determined using the published extinction coefficient of 3270 M⁻¹ cm⁻¹ (12). One dialysis tube was added to each vial, and the vials were placed into a shaker incubator and gently shaken for 16 h at room temperature. After dialysis, the concentration of free AIR was determined by the absorbance at 250 nm using the extinction coefficient listed above. The concentration of bound AIR was determined by subtracting the amount of free AIR from the total amount of AIR added at the beginning of the experiment. Control experiments conducted in the absence of enzyme revealed no change in AIR concentration, indicating that the substrate was stable over the course of the experiment. Control experiments of the enzymes in the absence of substrate revealed that they lost 10–20% of their activity during shaking at room temperature over the 16 h period. The dissociation constants and enzyme concentrations were determined by fitting the data from a plot of the total amount of AIR versus the amount of bound AIR to eq 1

\[
[AIR_{\text{bound}}] = \frac{E_T + AIR_T + K_d - \sqrt{(E_T + AIR_T + K_d)^2 - 4E_TAIR_T}}{2E_T}
\]

where \(E_T\) is the total enzyme concentration, \(AIR_T\) is the total AIR concentration, \(AIR_{\text{bound}}\) is the amount of AIR bound to the enzyme, and \(K_d\) is the dissociation constant. Reported errors are those generated from the curve fit.

RESULTS AND DISCUSSION

Structure of N⁵-CAIR Synthetase Complexed with Mg₂ATP. N⁵-CAIR synthetase from the bifunctional ADE2-encoding protein was cloned, expressed, purified, and crystallized as described in Materials and Methods. It is a dimer with overall dimensions of \(\sim 90 \times 80 \times 60 \) Å and a total buried surface area of 3600 Å². Each subunit of the dimer folds into three domains that are referred to as A, B, and C and that extend from Met 1 to Ala 119, from Glu 120 to Trp 183, and from Ala 184 to Arg 382, respectively (Figure 1). The A domain is characterized by a five-stranded parallel \(\beta\)-sheet flanked on each side by two \(\alpha\)-helices. Two additional \(\alpha\)-helices, oriented at approximately 90° with respect to one another and connected by Asn 103, provide a bridge from the A domain to the B domain. This helix—residue—helix motif is a structural hallmark for enzymes belonging to the ATP-grasp superfamily. The B domain contains a four-stranded antiparallel \(\beta\)-sheet, which is covered on one side by two \(\alpha\)-helices. The C domain is the most complicated of the three motifs with a total of nine \(\beta\)-strands that form a decidedly twisted antiparallel \(\beta\)-sheet. Whereas the A and C domains contribute to the subunit—subunit interface, the B domains splay away from the main body of the molecule, and the temperature factors for the residues forming this domain are typically higher than those found in the A and C domains. There are three residues that adopt cis-peptide bonds, Pro 93 in the A domain, Pro 142 in the B domain, and Pro 222 in the C domain. None of these is located near the active site.

The crystals used in this investigation contained two dimers in the asymmetric unit, and the \(\alpha\)-carbons for the four subunits superimpose with root-mean-square deviations (rmsds) as low as...
0.24 Å or as high as 0.98 Å. These differences result from variations in the orientations of the B domains relative to the A and C domains. Overall, the quality of the electron density was best for subunit 2 in the X-ray coordinate file, and thus, the following discussion refers only to it. Electron density corresponding to the bound ATP is displayed in Figure 2a. As can be seen, two magnesium ions accompany the ATP in the active site. One of these is wedged between an α- and a γ-phosphoryl oxygen, whereas the other is situated between a β- and a γ-phosphoryl oxygen. The ribose of the nucleotide adopts the C30-endo pucker.

A close-up view of the active site is presented in Figure 2b. With the exception of Lys 104, all of the residues responsible for binding the nucleotide are contributed by the B and C domains. Both magnesium ions are octahedrally coordinated with metal–ligand bond distances ranging from 1.8 to 2.4 Å. Magnesium ion “A” is surrounded by Glu 254, Glu 267, two phosphoryl oxygens, and a water molecule. Glu 254 functions as a bidentate ligand. Magnesium ion “B” is ligated by Glu 267, two phosphoryl oxygens, and two waters. Glu 267 not only functions as a bidentate ligand but also serves to bridge the two cations together. The metal binding geometry observed for the A. clavatus N5-CAIR synthetase is nearly identical to that previously reported for the enzyme from E. coli (18). Two lysine residues, namely, Lys 104 and Lys 146, provide additional positive charges to further neutralize the negatively charged phosphoryl groups of ATP. The adenine ring is anchored into the active site by hydrogen bonding with two side chains, Lys 146 and Glu 181, and with the carbonyl oxygen of Lys 182 and the backbone amide nitrogen of Ala 184. There is an additional hydrogen bond between the adenine ring and a water molecule. Both the 2- and 3-hydroxyls of the ribose lie within hydrogen bonding distance to the carboxylate of Glu 189. The indole ring of Trp 183 forms a T-shaped stacking interaction with the adenine ring.

**Structure of N5-CAIR Synthetase Complexed with MgADP and AIR.** As in the case of the structure analysis of N5-CAIR synthetase complexed with Mg2ATP, the crystals used to trap MgADP and AIR contained two dimers in the asymmetric unit. The four individual subunits correspond with root-mean-square deviations between 0.25 and 1.5 Å. Again, these differences arise from the flexibility of the B domains relative to the main body of the enzyme. In this X-ray analysis, subunit 3 displayed the best overall electron density, and thus, the following discussion refers only to it.

As shown in Figure 3a, the electron densities for ADP and AIR are very well ordered. Only one magnesium ion is observed lying between an α- and a β-phosphoryl oxygen of ADP. The AIR ribose adopts the C3-endo pucker. A distance of ~8 Å
separates the two ligands. The position of the B domain in the MgADP$^3$AIR complex moves slightly towards a more closed conformation than that observed for the Mg$^2$ATP complex. Overall, however, the polypeptide chains for the two enzyme complexes presented here are very similar and correspond with a root-mean-square deviation of 1.1 Å.

A close-up view of the active site is presented in Figure 3b. As might be expected, the hydrogen bonding patterns surrounding the adenine ring and the ribose of the ADP ligand are similar to those observed for ATP. The sole magnesium ion is octahedrally coordinated by two phosphoryl oxygens, two water molecules, and the side chains of Glu 254 and Glu 267. Glu 254 no longer functions as a bidentate ligand. Metal-ligand bond distances range from 1.9 to 2.4 Å.

The 5-aminoimidazole moiety of AIR is firmly anchored into the active site via hydrogen bonding interactions with three water molecules and the carboxylate group of Asp 153. One of these waters serves as a bridge between the 5-aminoimidazole moiety, the ribose ring oxygen, and the bridging phosphoryl oxygen. Glu 73 forms a hydrogen bond to the 2- and 3-hydroxyls of the ribose, whereas the positively charged side chains of Arg 155, Lys 345, and Arg 352 participate in electrostatic interactions with the phosphoryl group. Tyr 152 also provides a hydrogen bond to a phosphoryl oxygen of the AIR ligand.

**Analysis of Site-Directed Mutant Proteins of N$^5$-CAIR Synthetase.** The availability of the MgADP·AIR structure provided us with the necessary information to begin to assess the function of amino acids located in the active site of the enzyme. The investigations reported here focused on the AIR binding site and in particular targeted five residues (Glu 73, Tyr 152, Asp 153, Arg 155, and Lys 353) for site-directed mutagenesis and analysis. Note that Lys 353 does not directly hydrogen bond to AIR but rather lies within 2.6 Å of the carboxylate of Asp 153 (Figure 3b).
Each residue was mutated to alanine, and the resulting proteins were analyzed by steady-state kinetics and equilibrium dialysis. The results of these studies are listed in Table 3.
The kinetic parameters for the wild-type enzyme revealed that all of the mutations decreased the catalytic proficiency of the enzyme by 50–8000-fold. The $K_m$ for AIR for the mutant proteins increased, yet surprisingly, for most, the $K_d$ for AIR remained either unchanged or decreased. While there are kinetic reasons why $K_m$ and $K_d$ may not be equivalent, we expected that mutations in the substrate binding site would weaken the binding of AIR. The most likely explanation for the lower $K_d$ value is that for some mutant proteins, AIR can adopt nonproductive conformations in the active site, which generated additional binding contacts at the expense of catalysis. The equilibrium dialysis experiments also indicated that for all proteins, the active enzyme concentration (based upon curve fitting using eq 1) in the dialysis experiment was 50–80% lower than the total concentration of protein added. This indicates that either not all of the protein in solution existed in the proper conformation for substrate binding or multiple substrate molecules bind to the proteins in the absence of ATP. We are currently conducting additional experiments to clarify this result.

Mutation of Tyr 152 resulted in an increase in the $K_m$ and $K_d$ values. This residue is of particular interest given the recent discovery that substitution of a tyrosine at the appropriate location in the E. coli purT-encoded glycaminide ribonucleotide transformylase confers $N^5$-CAIR synthetase activity to the enzyme (23). As noted above, Tyr 152 forms a hydrogen bond to the phosphate group of AIR and also stacks with its imidazole ring. These interactions serve to lock the substrate into a productive conformation needed for catalysis and likely enhance the specificity of $N^5$-CAIR synthetase for an aromatic substrate.

The Glu 73 mutant is also of interest. This residue hydrogen bonds to the 2- and 3-hydroxyl groups of the AIR ribose and is distal to the site of catalysis. Thus, we were surprised to observe a decrease in $K_d$ and a 1400-fold decrease in $k_{cat}$ compared to those of the wild-type enzyme. An examination of the MgADP-AIR structure reveals no direct contact with any residue believed to be involved in catalysis. Glu 73, however, is in the same loop as Glu 75 which hydrogen bonds to Arg 271. Arg 271 is located in the putative catalytic region of the active site, and as discussed below, we believe that Arg 271 plays an important role in carboxyphosphate binding and in catalysis of this intermediate. Thus, in the presence of a substrate, Glu 73 could possibly stabilize this loop and thus align Arg 271 for catalysis.

One of the goals of this study was to identify the active site base required for catalysis. Our previous work, based upon a model of the substrate-binding site in the E. coli enzyme, suggested that either Asp 153 or Lys 353 could function as the base. The data presented here reveal that Lys 353 is ~4 Å from the exocyclic amine of AIR, whereas Asp 153 is only 3 Å distant. Site-directed mutagenesis, steady-state kinetic analysis, and equilibrium dialysis support this conclusion. Replacement of Asp with alanine results in an enzyme with essentially the same $K_m$ and $K_d$ as the wild-type enzyme, but with a $k_{cat}$ value more than 1600-fold lower. Clearly, Asp 153 is involved in catalysis, but not substrate binding. Mutation of Lys 353 to alanine also generated an inactive enzyme. The mutant protein, however, was capable of binding AIR with the same affinity as the wild-type enzyme. On the basis of our structural information, we suggest that Lys 353 is the protein inhibitor of AIR synthetase activity to the enzyme.

Implications for the Catalytic Mechanism. $N^5$-CAIR synthetase is believed to catalyze the conversion of AIR into $N^5$-CAIR by the use of the unstable, high-energy intermediate carboxyphosphate (13). Once carboxyphosphate has been generated from ATP and bicarbonate, its role in the remainder of the reaction is uncertain. Either it is directly attacked by AIR, or it decomposes to generate CO2, which then reacts with AIR to generate $N^5$-CAIR. Previously, we proposed a mechanism for $N^5$-CAIR synthetase based upon the structure of the E. coli enzyme complexed with MgADP and P$_i$ (18). This mechanism relied upon molecular modeling to identify the location of the AIR binding site. Importantly, the structural information presented in this paper correlates exceedingly well with the originally proposed model.

The site-directed mutagenesis data presented here now allow us to add new detail to our published catalytic mechanism by assigning roles for individual amino acids in both substrate binding and catalysis as highlighted in Scheme 2. Specifically, the catalytic mechanism begins with the reaction of bicarbonate and ATP to generate carboxyphosphate and ADP. Molecular mechanics calculations suggest that the carboxyphosphate intermediate is more stable in the pseudochair conformation (18) as
drawn in Scheme 2. By overlaying the model of the *E. coli* MgADP·P·ARP synthetase complex with the *A. clavatus* MgADP·AIR structure, we generated a possible location for the carboxyphosphate intermediate, based upon the location of inorganic phosphate (I8) (Figure 4a). On the basis of this modeling, it appears that the carboxyphosphate interacts with both Arg 271 and Lys 353. Note, however, that in this model the distance between the exocyclic amine of AIR and the carbonyl carbon of carboxyphosphate is 3.6 Å and the angle of approach is incorrect, thus suggesting that AIR does not directly attack carboxyphosphate. Instead, we suggest that carboxyphosphate decomposes to CO$_2$ via an intramolecular proton abstraction by the phosphate group (Scheme 2). This deprotonation and subsequent release of HPO$_4^{2-}$ is aided by both Arg 271 and Lys 353. Molecular modeling reveals that the CO$_2$ generated is directly positioned between Arg 271 and Lys 353, where each residue could form a hydrogen bond to the oxygen atoms of CO$_2$ (Figure 4b). These residues, which likely are positively charged, could also serve to enhance the electrophilicity of CO$_2$ by drawing electrons away from the carbon atom. An examination of the model presented in Figure 4b indicates that CO$_2$ is ~2.7 Å from the amine of AIR, and there is an optimal alignment between the amine and the carbon of CO$_2$. Catalysis occurs in a concerted fashion as the amine of AIR attacks the carbon of CO$_2$ and Asp 153 removes its proton.
The resulting negative charge on the oxygen atom is stabilized by either Arg 271 or Lys 353 (Scheme 2).

In summary, the results presented here provide, for the first time, detailed molecular information regarding the binding of AIR to N\(^5\)-CAIR synthetase. Furthermore, this study is the first to present structural information on any N\(^5\)-CAIR synthetase isolated from lower eukaryotes. Such knowledge has given us the ability to rationally select amino acids for mutation, and we have demonstrated that both Asp 153 and Lys 353 are critical for catalysis. These data provide a more detailed understanding of the enzyme, which in turn, will help in the design of novel antibacterial and antifungal agents.

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REFERENCES


