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The "Super Mutant" of Yeast FMN Adenylyltransferase Enhances the Enzyme Turnover Rate by Attenuating Product Inhibition

Carlos Huerta,[†,§] Nick V. Grishin,[‡,†] and Hong Zhang*[,†]

[†]Department of Biophysics and Department of Biochemistry and [‡]Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75390, United States

Supporting Information

ABSTRACT: FMN adenylyltransferase (FMNAT) is an essential enzyme catalyzing the last step of a two-step pathway converting riboflavin (vitamin B2) to FAD, the ubiquitous flavocoenzyme. A structure-based mutagenesis and steady-state kinetic analysis of yeast FMNAT unexpectedly revealed that mutant D181A had a much faster turnover rate than the wild-type enzyme. Product inhibition analysis showed that wild-type FMNAT is strongly inhibited by FAD, whereas the D181A mutant has an attenuated product inhibition. These results provide a structural basis for the product inhibition of the enzyme and suggest that product release may be the rate-limiting step of the reaction.

Flavocoenzymes, including flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are versatile redox cofactors involved in many fundamental cellular processes in all living organisms. They participate in energy production, metabolism, light sensing, DNA repair, chromatin remodeling, protein folding, neural development, and circadian rhythm regulation.[1−4] In mammals, FAD is synthesized from riboflavin (vitamin B_2) obtained from the diet[5,6] via two enzymatic steps catalyzed by riboflavin kinase (RFK, EC 2.7.1.26) and FMN adenylyltransferase (FMNAT, EC 2.7.7.2). Phosphorylation of riboflavin by RFK is crucial for specific absorption of the vitamin and is the physiologically rate-limiting step in the biosynthesis of flavocoenzymes,[7−9] whereas product (FAD) feedback inhibition was observed for mammalian FMNAT, suggesting that biosynthesis of FAD is also regulated at the FMNAT reaction step.[10,11]

We have previously determined crystal structures of a eukaryotic FMNAT from a yeast species *Candida glabrata* (*Cg*FMNAT) and its complexes with substrates (FMN and ATP) and products (FAD and pyrophosphate).[12] Eukaryotic FMNAT contains a core domain with a modified Rossman-fold topology and a C-terminal extension.[12−14] The substrate binding and catalytic site is located at the interface of the two domains. Most prominently, the isoalloxazine ring of the FMN substrate binds to one face of the central β-sheet on the same side as the adenine moiety of ATP, constituting a novel flavin binding site unseen in any other flavin binding proteins.[12,15] Steady-state kinetics analysis of *Cg*FMNAT showed that although the enzyme apparently binds its substrates with high affinity (the K_m's for FMN and ATP are 0.76 ± 0.15 and 10.7 ± 2.3 μM, respectively), the overall turnover rate is very slow (k_{cat} of 0.087 s^{-1}). Previous kinetic studies of endogenous mammalian FMNAT indicated that the enzyme is product-inhibited, and the K_i values of FAD (0.75−1.30 μM) are close to the estimated free FAD concentration (0.4 μM) in the cell.[10] It has been suggested that biosynthesis of FAD is regulated at the FMNAT reaction step through a product feedback inhibition mechanism.[10]

In this study, we conducted structure-based mutagenesis and kinetics analysis of *Cg*FMNAT to investigate the role of a number of active site residues and the catalytic mechanism of eukaryotic FMNAT. The crystal structures of *Cg*FMNAT revealed details of the unique active site arrangement and substrate binding mode of the enzyme[12,16] (Figure 1A). Several structural elements, collectively termed the "flavin motif", are involved in flavin ligand binding. In particular, the isoalloxazine ring is sandwiched between the indole ring of Trp184 and the guanidinium group of Arg189, while the hydrophilic pyrimidine ring forms two specific hydrogen bonds with the main chain amide and the side chain of Asp181. Additionally, Asn62, Asp66, Asp168, and Arg297 are found to interact with ATP phosphate groups or to coordinate the catalytic Mg^{2+} ion either directly or indirectly through water molecules (Figure 1A).

On the basis of these structural observations, we selected a set of active site residues for mutagenesis studies to investigate their roles in substrate binding and/or catalysis (Table S1 of the Supporting Information). Under the wild-type (wt) *Cg*FMNAT saturation condition, the specific activities of the mutants were determined and are summarized in Table S2 of the Supporting Information. Mutation of the PP-loop motif residue Asn62 to either Ala or Ser resulted in a moderate decrease in activity, while mutation of the Mg^{2+} ion coordinating Asp66 to an Ala resulted in a practically inactive enzyme, indicating the essential role of Asp66 in directly ligating to the catalytic Mg^{2+} ion. Similar to that of D66A but to a lesser extent, the specific activity of the D168A mutant is reduced by >90%, indicating that the interaction with the two Mg^{2+} ion-coordinating waters by Asp168 is important for the catalytic activity of the enzyme. Interestingly, mutations of the two Arg residues, Arg297 and Arg300 near the C-terminus of the protein, produced opposite results. While the specific activity of R300A is reduced to only ~7% of that of the wt enzyme, the R297A mutant is nearly twice as active, indicating that both residues are involved in the enzyme reaction but in different ways.

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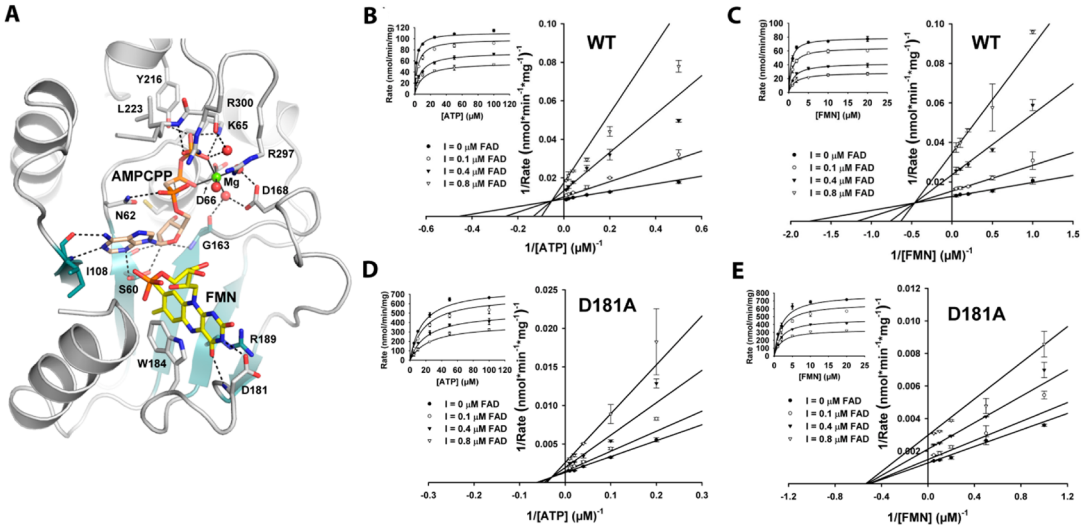

Figure 1. Structure-based mutagenesis and product inhibition analysis of *Cg*FMNAT. (A) Active site configuration and interactions with substrate FMN and ATP analogue AMPCPP. The bound Mg^{2+} ion is shown as a green sphere, and water ligands are shown as red spheres. Hydrogen bonds are shown as dashed lines and metal ligand interactions as solid lines. (B and C) Steady-state kinetics of product inhibition of wt *Cg*FMNAT by FAD against ATP and FMN. (D and E) Steady-state kinetics of product inhibition of the *Cg*FMNAT D181A mutant by FAD against ATP and FMN. The rates were globally fitted to the velocity equation for general inhibition mode (eq 2 of the Supporting Information).

To explore roles of the flavin motif residues in FMN substrate binding, we designed both W184A and D181A mutants to weaken the interaction with FMN and expected them to have higher $K_{m,FMN}$ values. These mutants have even more dramatic and unexpected outcomes. The loss of the stacking interaction with the isoalloxazine ring of FMN in the W184A mutant reduced the specific activity to ~13% of that of the wt enzyme. However, the specific activity of D181A is ~8-fold higher than that of wt *Cg*FMNAT. Due to the fact that D181A and R297A have increased specific activity, both are termed "super mutants". Lastly, removing the last three positively charge lysine residues (Δ3K) resulted in no appreciable difference in specific activity, indicating that these residues are not involved in substrate binding or catalysis.

To understand the effect of active site mutants on the substrate binding and turnover rate (k_{cat}) of *Cg*FMNAT, apparent steady-state kinetic parameters were determined for all mutants except for D66A, which had no measurable activity. The results are listed in Table 1. As predicted, the N62S mutant has a decreased k_{cat} (0.017 s^{-1} vs 0.89 s^{-1}) accompanied by an increased $K_{m,ATP}$ (from 2.0 to 5.7 μM), while the $K_{m,FMN}$ of this mutant is unchanged, consistent with the role of N62 in ATP binding (Figure 1). Interestingly, the N62A mutant has a lower apparent $K_{m,ATP}$ (0.5 μM) than wt *Cg*FMNAT (2.0 μM), which was unexpected, as Asn62 interacts with ATP and the N62A mutation was predicted to increase the apparent $K_{m,ATP}$. It is possible that while the N62A mutant is able to bind ATP tightly, the conformation of the ATP may not be as optimal as that bound to the wt enzyme, resulting in a slightly slower k_{cat} (0.042 s^{-1} vs 0.089 s^{-1}). The D168A mutant has a significantly decreased k_{cat} and a much higher apparent $K_{m,ATP}$ (Table 1), consistent with its role in coordinating the catalytic metal ion and ATP binding (Figure 1A). Arg297 was predicted to interact with either ATP or FMN phosphates.[12] The apparent $K_{m,ATP}$ and $K_{m,FMN}$ of R297A mutant increased ~5- and ~3-fold, respectively (Table 1), supporting such a proposal. Despite an increase in the apparent K_m's, the R297A mutant has a k_{cat} 2 times faster than that of wt *Cg*FMNAT.

Table 1. Steady-State Kinetic Parameters of wt *Cg*FMNAT and Its Mutants

	$K_{m,ATP}$ (μM)	$K_{m,FMN}$ (μM)	k_{cat} (s^{-1})
wt	2.0 ± 0.3	1.0 ± 0.4	0.089
N62A	0.5 ± 0.2	<0.50	0.042
N62S	5.7 ± 0.9	1.1 ± 0.1	0.017
D168A	28.1 ± 7.2	7.1 ± 2.2	0.017
D181A	6.4 ± 0.4	1.5 ± 0.4	0.88
W184A	23.6 ± 4.6	199.4 ± 27.9	0.26
R297A	10.3 ± 1.6	3.0 ± 0.5	0.21
R300A	68.3 ± 11.4	2.3 ± 0.6	0.010
R297A/R300A	123.5 ± 24.0	9.3 ± 2.2	0.012

For the flavin motif mutants, FMN binding of W184A mutant is significantly weakened as demonstrated by a 200-fold increase of $K_{m,FMN}$. The $K_{m,ATP}$ of the mutant is also increased (from 2.0 to 24 μM), indicating that ATP binding is also affected. Unexpectedly, the k_{cat} of the W184A mutant is not affected and even increased by ~3-fold. Most surprisingly, the D181A mutant has a much increased apparent k_{cat}, ~10 times that of wt *Cg*FMNAT, and a relatively unchanged apparent $K_{m,FMN}$. It appears that Asp181, though observed to interact with FMN or FAD at the outer edge of the isoalloxazine ring, does not contribute to the initial binding of the FMN substrate but may act as a "gatekeeper" to stabilize enzyme-bound FMN, as well as product FAD.

Because Asp181 is involved in the interaction with FAD or FMN and is distant from the catalytic site of the enzyme where adenylyl transfer occurs, we hypothesized that the rate-limiting step of *Cg*FMNAT could be product release, and the D181A mutant would have a less pronounced product inhibition and, thus, a faster turnover rate. To test this hypothesis, we investigated the effect of FAD product on the initial rates. The results show that wt *Cg*FMNAT is strongly inhibited by the product FAD. The K_i is 0.10 μM against ATP and 0.12 μM against FMN (Figure 1B,C). In the case of super mutant D181A, the K_i values of FAD against ATP and FMN are 0.39 and 0.67 μM (Figure 1D,E), respectively, which are markedly

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higher than that of the wt enzyme. Fitting of the general inhibition equation gives an α of 8.6, suggesting that FAD acts as a mixed-type inhibitor with respect to both ATP and FMN.

These results revealed several interesting aspects of *Cg*FMNAT that were not obvious from the crystal structures. First, the binding of the two substrates ATP and FMN appears to be synergistic. For example, mutating residue Asp168 disrupted the coordination of the catalytic Mg^{2+} ion, which leads to a significant increase in $K_{m,ATP}$ and a decrease in k_{cat}. Although Asp168 does not interact with the FMN substrate directly, the $K_{m,FMN}$ also increased 7-fold, indicating that FMN substrate binding is affected by this mutation as well. Conversely, residue Trp184 stabilizes the bound FMN through stacking interaction and does not make any direct contact with the ATP substrate. While mutating Trp184 to Ala severely compromised FMN binding as the $K_{m,FMN}$ increased 200-fold, the $K_{m,ATP}$ of the mutant also increased >10-fold. Apparently, the effect of the W184A mutation on the binding of ATP is indirect and likely a consequence of destabilization of FMN. From the structure of the ternary *Cg*FMNAT−substrate complex, the binding sites for ATP and the isoalloxazine ring of FMN form a continuous channel.[12] The C8 methyl group of FMN is in direct contact with the adenine ring of ATP (Figure 1A), providing additional stabilizing interactions for ATP. Likewise, the bound ATP molecule forms part of the isoalloxazine ring binding pocket and would restrict its potential movement.

The crystal structure of the *Cg*FMNAT−product complex revealed the proximity of the Arg297 side chain to both phosphates of the FAD product, which comes from the ATP α-phosphate and FMN phosphate.[12,16] We speculate that Arg297 could be involved in the interaction with the phosphate groups of both substrates and helps in their positioning for the nucleophilic attack in the adenylyl transfer reaction. Additionally, the crystal structure of the *Saccharomyces cerevisiae* FMNAT·FAD complex[16] indicates that the nearby Arg300 (Arg303 of *S. cerevisiae* FMNAT) could also be involved in the interaction with the ATP phosphates in a manner similar to that observed in bacterial adenosine 5′-phosphosulfate (APS) reductase.[17] Steady-state kinetics analysis supports the involvement of Arg297 and Arg300 in binding ATP, probably through positioning the α- or/and β-phosphate for the adenylyl transfer reaction. Interestingly, although the R297A mutant has slightly weaker affinities for both ATP and FMN, its k_{cat} is ∼2-fold faster than that of the wt enzyme, suggesting that it also likely plays a role in the release of the FAD product.

The most unexpected results are obtained with the D181A mutant. In wt *Cg*FMNAT, Asp181 forms two hydrogen bonds with the hydrophilic pyrimidine ring of the bound flavin (Figure 1A). There is no conformational difference between wt *Cg*FMNAT and the D181A mutant (see the Supporting Information and Table S3) except that the loss of the Asp181 side chain eliminated one of these two hydrogen bonds. However, the D181A mutation results in no appreciable change in the apparent $K_{m,FMN}$. Instead, the k_{cat} of D181A increased ∼10-fold. Because Asp181 is distant from the catalytic site where adenylyl transfer occurs and does not markedly affect substrate binding, we propose that the D181A mutant would affect product release and that product release is the rate-limiting step of the *Cg*FMNAT-catalyzed reaction. Indeed, product inhibition analysis showed that product inhibition in the D181A mutant is significantly weakened compared to that in wt *Cg*FMNAT. Our data suggest that Asp181 plays a role primarily in regulating FAD product release rather than FMN substrate binding.

■ ASSOCIATED CONTENT

Supporting Information

Details of the experimental procedures and Tables S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: zhang@chop.swmed.edu. Phone: (214) 645-6372. Fax: (214) 645-5948.

Present Address

[§]Department of Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390.

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Notes

The authors declare no competing financial interests.

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