"Super mutant" of yeast FMN adenylyltransferase enhances enzyme turnover rate by attenuating product inhibition

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Supporting Information

MATERIAL AND METHODS

Mutagenesis, protein expression and purification

*Cg*FMNAT mutants N62A, N62S, D66A, D168A, D181A, W184A, R297A, R300A and the deletion of the last three lysines (Δ3K) were generated using QuikChange™ site-directed mutagenesis procedure (Invitrogen) on the template vector pHIS parallel containing the wt *Cg*FMNAT coding sequence (*1*). Incorporation of the desired mutant constructs were confirmed by DNA sequencing (Supplementary Material **Table S1**).

For large scale protein expression and purification, the expression vectors harboring mutant *Cg*FMNAT DNA were transformed into *E. coli* BL21(DE3) (Novagen) and cells were grown in 1 L Luria-Bertani (LB) broth containing 100 μg/ml ampicillin. Cultures were grown at 37 °C to an absorption of 0.6 at 600 nm and induced overnight at 20 °C by the addition of 0.8 mM isopropyl β-D-thiogalactoside. Cells were harvested by centrifugation, resuspended in the lysis buffer (20 mM HEPES, pH 8.0, 100 mM NaCl, 0.03% Brij-35 and 10% glycerol) supplemented with 2 mM PMSF and 1 mM DTT, and completely lysed by sonication. Clarified cell lysate was loaded on a nickel-sepharose affinity column (GE Healthcare) equilibrated with Buffer A (20 mM HEPES, pH 8.0, 300 mM NaCl, 20 mM imidazole, 5% glycerol and 1 mM DTT) and the His_6-tagged *Cg*FMNAT was eluted with a 20–500 mM imidazole gradient. The His_6-tag was then cleaved with TEV protease during overnight dialysis to a buffer containing 20 mM HEPES, pH 7.5, 50 mM NaCl, 5% glycerol and 1 mM DTT at 4 ºC. The protein with removed His_6-tag was recovered by passing the sample through the nickel-sepharose column a second time. The tag-free *Cg*FMNAT was loaded onto a Resource Q anion exchange column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, 5% glycerol and 1 mM DTT, and eluted with a 0−350 mM NaCl gradient. To remove the intrinsically bound flavin, the protein

was incubated with 1.5 M ammonium sulfate and loaded onto a phenyl-sepharose hydrophobic-interaction chromatography column equilibrated with 20 mM HEPES, pH 7.5 and 1.5 M ammonium sulfate. The protein was eluted with a 1.5 M–0 M ammonium sulfate gradient. The purified protein was then dialyzed into a buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl and 1 mM DTT. The phenyl-sepharose chromatography step was omitted for D181A and W184A mutants, as the protein contained no detectable flavin. For *Cg*FMNAT D181A crystallization, the mutant protein was purified using a similar procedure with a final Superdex 75 size exclusion chromatography column following the Resource Q column. Detection of flavin was measured by a UV/visible wavelength scan. Protein purity was monitored by SDS-PAGE and the protein concentration was determined by Bio-Rad protein assay.

Steady-state kinetics analysis

A continuous spectrophotometric assay that couples the release of the product inorganic pyrophosphate (PP_i) to the generation of 2-amino-6-mercapto-7-methylpurine (*2*, *3*) was used. To measure the specific activity (nmole/min/mg protein), the reaction mixture (0.25 mL) contained 20 mM HEPES, pH 7.5, 2 mM magnesium chloride, 10 units purine nucleoside phosphorylase, 1 unit inorganic pyrophosphatase (PP_iase), 0.2 mM 2-amino-6-mercapto-7-methylpurine riboside, 20 μM FMN, 100 μM ATP and 21 nM of *Cg*FMNAT protein. Protein concentrations used were: 40 nM for N62A, 80 nM for N62S, 160 nM for D168A, 7 nM for D181A, 300 nM for W184A, 15.8 nM for R297A, 87.5 nM for R300A, and 110 nM for Δ3K. To obtain the apparent $K_{m,\ FMN}$, ATP was held at a saturating concentration of 100 μM for native *Cg*FMNAT, D181A and R297A, and 400 μM for the remaining *Cg*FMNAT mutants, while FMN was varied between 1-800 μM. To obtain the apparent $K_{m,\ ATP}$, FMN was held at a saturating concentration of 40 μM for D168A and R300A; 400 μM for W184A and 20 μM for

the remaining *Cg*FMNAT mutants, while ATP was varied between 1-1000 μM. All reactions were carried out in duplicates or triplicates and performed at 25 °C. The reaction was initiated by the addition of *Cg*FMNAT enzymes, and the progress was monitored by measuring the absorption at wavelength 355 nm for 10 minutes. For native and mutant *Cg*FMNATs the apparent steady-state kinetic parameters were determined by fitting the initial rates to the general Michaelis-Menten equation (1) (Eq. (1)).

$$v = \frac{V_{max,app}[S]}{(K_{m,app} + [S])} \quad (1)$$

$K_{m,app}$ is the apparent Michaelis-Menten constant, and [S] is the concentration of ATP when FMN is at saturated concentration or FMN when ATP is at saturated concentration. A nonlinear least-squares method as implemented in Sigma Plot was used to fit the data.

Product inhibition analysis

The malachite green end-point assay was utilized to measure product inhibition by FAD to avoid the potential interference from the flavin absorption (*4-6*). To determine the effect of FAD on the initial rates, three different time points were collected within the linear range of the reaction for the wt and D181A mutant. The reaction mixture (0.3 ml) contained 20 mM HEPES, pH 7.5, 2 mM magnesium chloride, 0.06 units inorganic pyrophosphatase (PPase), 21 nM *Cg*FMNAT or 5 nM D181A, and appropriate amount of ATP, FMN, and FAD. In the reaction mixture, the concentration of the fixed substrate ATP or FMN was 6 μM and 4 μM for *Cg*FMNAT, and 50 μM and 6 μM for D181A, respectively. Inhibition by the product was conducted at FAD concentrations of 0, 0.1, 0.4 and 0.8 μM. For product inhibition with respect to ATP, the FMN concentration was held constant while the ATP concentration was varied between 2-100 μM for native FMNAT and 5-100 μM for D181A mutant. For product inhibition with respect to FMN, the ATP concentration was held constant while the FMN concentration

was varied between 1-20 μM. *Cg*FMNATs were pre-incubated with the appropriate concentration of FAD for 30 minutes on ice before adding to the reaction mixture to initiate the reaction. The reaction was allowed to progress for 1 to 18 minutes, and was stopped by the addition of 300 μL malachite green reagent. The color development was quenched after 2 minutes with 60 μL of quenching solution and incubated for 20 minutes at 25 °C before measuring the absorbance at wavelength 650 nm. As a blank, *Cg*FMNAT enzyme was left out of the reaction mixture.

The stock solutions for the assay were prepared as following (*5, 6*): reagent A, 41.9 mM ammonium molybdate in ddH_2O; reagent B, 3.1 M H_2SO_4; reagent C, 1.13 mM malachite green in ddH_2O; and reagent D, 0.660 % poly (vinyl alcohol) in ddH_2O. The malachite green reagent was prepared by mixing the reagents to provide a final solution of 12.04 mM ammonium molybdate, 0.9 M H_2SO_4, 0.24 mM malachite green, 0.14 % poly (vinyl alcohol). The malachite green reagent was filtered using a 0.2 μm syringe filter. A quenching solution was prepared with 40% (w/v) trisodium citrate dihydrate. All reagents were stored at 4ºC.

The effect of product inhibition by FAD on the initial rates of wt and D181A *Cg*FMNAT were fitted to the general inhibition equation (2) (commonly referred to as mixed-type inhibition) for readily reversible inhibitors (*7, 8*) (Eq. (2)).

$$\nu = \frac{V_{max}[\mathrm{S}]}{\left(K_m(1+[\mathrm{I}]/K_i)+[\mathrm{S}](1+[\mathrm{I}]/\alpha K_i)\right)} \quad (2)$$

where K_i is the inhibition constant, α is a quantitative parameter that provides a measure of the type of inhibition (*7, 8*), and [I] is the concentration of the inhibitor (FAD). A nonlinear least-squares method as implemented in the Sigma Plot Enzyme Kinetics module was used to fit the data globally.

Crystallization and structure determination of D181A mutant

The purified D181A mutant protein was concentrated to 21 mg/mL. The crystals were grown by mixing 1.5 μl of protein with 1.5 μl of reservoir solution composed of 0.1 M Na acetate, pH 4.4-5.4 and 6-12% (w/v) PEG 4000, and equilibrating against the reservoir at 16 ºC in a hanging-drop vapor diffusion setting. The reagents and greased 24-well plates were chilled on ice before setting up crystallization drops. All crystals were cryoprotected in a solution containing all the reservoir components and increments of glucose (10%, 20% and 30%), flash-frozen in liquid propane and stored in liquid nitrogen. The 1.7 Å dataset was collected in-house with X-ray from a rotating anode generator (Rigaku FRE SuperBright), recorded on an RAXIS IV (Rigaku) image plate detector. The data was processed with the HKL2000 package (*9*) and the phases were determined by the molecular replacement method using program Phaser (*10*) and the apo-*Cg*FMNAT structure (PDB ID: 3FWK) as the search model. The refinements were performed using REFMAC (*11*) and manual model building was performed with Coot (*12*). Model quality was assessed by MolProbity (*13*). Data collection and refinement statistics are presented in **Table S3**. The conformation of the D181A protein is essentially the same as the wt *Cg*FMNAT with a root mean square deviation of all C_α atoms to be 0.07Å. The atomic coordinates and structure factors have been deposited with the Protein Data Bank (PDB) under accession code 4KKV.

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Table S1. Primers used for *Cg*FMNAT mutagenesis

Mutant[a]	Primer Sequence[b]
N62A (AAT→GCT)	5'-GGTGAAATATCGTTTTCCTATGCTGGAGGCAAAGACTGCCAGGTG-3' 3'-CCACTTTATAGCAAAAGGATACGACCTCCGTTTCTGACGGTCCAC-5'
N62S (AAT→TCA)	5'-GGTGAAATATCGTTTTCCTATTCAGGAGGCAAAGACTGCCAGGTG-3' 3'-CCACTTTATAGCAAAAGGATAAGTCCTCCGTTTCTGACGGTCCAC-5'
D66A (GAC→GCT)	5'-CGTTTTCCTATAATGGAGGCAAAGCTTGCCAGGTGCTGTTATTACTATA-3' 3'-GCAAAAGGATATTACCTCCGTTTCGAACGGTCCACGACAATAATGATAT-5'
D168A (GAT→GCT)	5'-GTCATCGGTATAAGACACACTGCTCCATTTGGCGAGCATTTAAAG-3' 3'-CAGTAGCCATATTCTGTGTGACGAGGTAAACCGCTCGTAAATTTC-5'
D181A (GAT→GCT)	5'-AAGCCTATTCAAAAGACAGCTGCTAATTGGCCAGATTTC-3' 3'-TTCGGATAAGTTTTCTGTCGACGATTAACCGGTCTAAAG-5'
W184A (TGG→GCT)	5'-CCTATTCAAAAGACAGATGCTAATGCTCCAGATTTCTATCGTCTACAACCT-3' 3'-GGATAAGTTTTCTGTCTACGATTACGAGGTCTAAAGATAGCAGATGTTGGA-5'
R297A (AGA→GCT)	5'-GTACCTAGTCGATGACAAACTTGAAGCTGCAGGAAGAATTAAAAAGAAATGAG-3' 3'-CATGGATCAGCTACTGTTTGAACTTCGACGTCCTTCTTAATTTTTCTTTACTC-5'
Δ3K (AAA→TAA)	5'-TGAAAGAGCAGGAAGAATTTAAAAGAAATGAGTCGACGAGC-3' 3'-ACTTTCTCGTCCTTCTTAAATTTCTTTACTCAGCTGCTCG-5'

[a]Codons in parenthesis list the change from the native codon to the mutant codon.
[b]The forward and reverse primers for each mutant are listed with the mutagenesis site underlined.

Table S2. Specific activities of *Cg*FMNAT mutants

Protein Constructs	Mutant location	Functional Role	Percent Specific Activity
Native	n/a		95.7 ± 3.8
N62A	PP loop	ATP interaction	45.3 ± 2.8
N62S	PP loop	ATP interaction	26.2 ± 3.4
D66A	PP loop	Direct ligand to Mg^{2+}	2.2 ± 0.1
D168A	ARG1	Indirect ligand to Mg^{2+}	7.7 ± 7.3
D181A	Flavin	Isoalloxazine ring interaction	761.5 ± 33.7
W184A	Flavin	Isoalloxazine ring interaction	13.0 ± 5.6
R297A	ARG2	ATP and FMN phosphate interaction	171.0 ± 17.0
R300A	ARG2	ATP and FMN phosphate interaction	6.6 ± 6.9
R297,300A	ARG2	ATP and FMN phosphate interaction	5.9 ± 3.1
Δ last K's	ARG2		95.5 ± 10.1

Table S3. Crystallographic data and refinement statistics of *Cg*FMNAT D181A mutant

	D181A mutant
Data collection	
Space group	$P3_221$
Cell dimensions	
a, *b*, *c* (Å)	80.3, 80.3, 78.2
α, β, γ (°)	90, 90, 120
Resolution (Å)	50.00-1.74 (1.77-1.74)*
R_{sym} or R_{merge}	0.053 (0.497)
$I / \sigma I$	21.6 (1.26)
Completeness (%)	99.3 (95.4)
Redundancy	5.5 (3.5)
Refinement	
Resolution (Å)	24.4-1.74
No. reflections	28606
R_{work} / R_{free}	0.172/0.201
No. atoms	
Protein	2613
Water	332
Organic	12
Inorganic	1
B-factors	
Protein	21.6
Water	31.1
Organic	34.7
Inorganic	22.6
R.m.s. deviations	
Bond lengths (Å)	0.006
Bond angles (°)	1.082

*Values in parentheses are for highest-resolution shell.