Supplementary Information

Structural basis of improved second generation 3-nitro-tyrosine tRNA synthetases

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Description of selection plasmids

The library of aminoacyl-tRNA synthetases was encoded on a kanamycin (Kn) resistant plasmid (pBK, 3000 bp) under control of the constitutive *Escherichia coli* GlnRS promoter and terminator. The aminoacyl synthetase library (3D-Lib) was randomized as follows: Leu65, His70, Gln155, and Ile159 were randomized to all 20 natural amino acids; Tyr32 was randomized to 15 natural amino acids (less Trp, Phe, Tyr, Cys, and Ile); Asp158 was restricted to Gly, Ser, or Val; Leu162 was restricted to Lys, Ser, Leu, His, and Glu; and Phe108 and Gln109 were restricted to the pairs Trp-Met, Ala-Asp, Ser-Lys, Arg-Glu, Arg-Pro, Ser-His, or Phe-Gln. The library plasmid, pBK-3D-Lib, was moved between cells containing a positive selection plasmid (pCG) and cells containing a negative selection plasmid (pNEG) (*1*).

The positive selection plasmid, pCG (10000 bp), encodes a mutant *Methanocaldococcus jannaschii* (Mj) tyrosyl-$tRNA_{CUA}$, an amber codon-disrupted chloramphenicol acetyltransferase, an amber codon-disrupted T7 RNA polymerase that drives the production of green fluorescent protein, and the tetracycline (Tet) resistance marker. The negative selection plasmid, pNEG (7000 bp), encodes the mutant tyrosyl-$tRNA_{CUA}$, an amber codon-disrupted barnase gene under control of an arabinose promoter and rrnC terminator, and the ampicillin (Amp) resistance marker. pCG electrocompetent cells and pNEG electrocompetent cells were made from DH10B cells carrying the respective plasmids and stored in 100 μL aliquots at −80 °C for future rounds of selection.

The synthetase library in pBK-3D-Lib was transformed by electroporation into DH10B cells containing the positive selection plasmid, pCG. The resulting pCG/pBK-3D-Lib-containing cells were amplified in 1 L of 2×YT with 50 μg/mL Kn and 25 μg/mL Tet with shaking at 37 °C. The cells were grown to saturation, then pelleted at 5500 x g, resuspended in 30 mL of 2×YT and 7.5 mL of 80% glycerol, and stored at −80 °C in 1 mL aliquots for use in the first round of selections.

Expression and purification of the nitroTyr 5B RS

BL21(DE3) cells containing a *pET28* plasmid with the nitroTyr 5B gene inserted at the NcoI and XhoI sites (see Materials and Methods of main text) were grown at 37 ^{o}C in 1 L of LB media supplemented with 50 μg/ml kanamycin media to an OD_{600} ~0.6 – 0.8, at which time expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. After 18 h, cells were pelleted, frozen in liquid nitrogen and stored at -80 ^{o}C.

The nitroTyr-5B RS was purified similarly to previous described.(*2*) All steps were carried out at 4 ^{o}C or on ice. Briefly, cells were resuspended in lysis buffer (50 mM HEPES, 500 mM NaCl, 10% glycerol, 5 mM imidazole, 0.1% Triton X-100 and 10 mM β-mercaptoethanol, pH 7.9), lysed by sonication, and centrifuged at 20,000 x g for 1 h at 4 ^{o}C. The supernatant was incubated with Ni-NTA agarose (Pierce) for 1 h with stirring, which was then washed with 10 column volumes of wash buffer (50 mM HEPES, 500 mM NaCl, 10% glycerol, 5 mM imidazole and 10 mM β-mercaptoethanol, pH 7.9) and eluted with wash buffer supplemented with 250 mM imidazole. The eluted protein was dialyzed overnight into 25 mM Tris, 25 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, pH 8.5 and applied to a Q-Sepharose Fast Flow (GE Healthcare) column pre-equilibrated in the same buffer. The column was washed with 5 column volumes of this same buffer, at which time a gradient from 25 mM to 1 M NaCl was applied over 10 column volumes. Fractions containing >95% pure nitroTyr-5B RS as determined by SDS-PAGE were pooled, dialyzed overnight into storage buffer (20 mM Tris, 50 mM NaCl, 10 mM β-mercaptoethanol, pH 8.5), concentrated to 20 mg/ml, frozen in liquid nitrogen and stored at -80 ^{o}C.

Tables.

Supplementary Table 1. Components for autoinducing and non-inducing mediums, for final volume of 500 mL.

	Autoinduction medium	Non-inducing medium	Autoinducing plates
5% aspartate, pH 7.5	25 mL	25 mL	25 mL
10% glycerol	25 mL	-	25 mL
25× 18 amino acid mix[a]	20 mL	20 mL	20 mL
50× M salts[a]	10 mL	10 mL	10 mL
leucine (4 mg/mL), pH 7.5	5 mL	5 mL	5 mL
20% arabinose	1.25 mL	-	1.25 mL
1 M $MgSO_4$	1 mL	1 mL	1 mL
40% glucose	625 µL	6.25 mL	125 µL
Trace metals[a]	100 µL	100 µL	100 µL

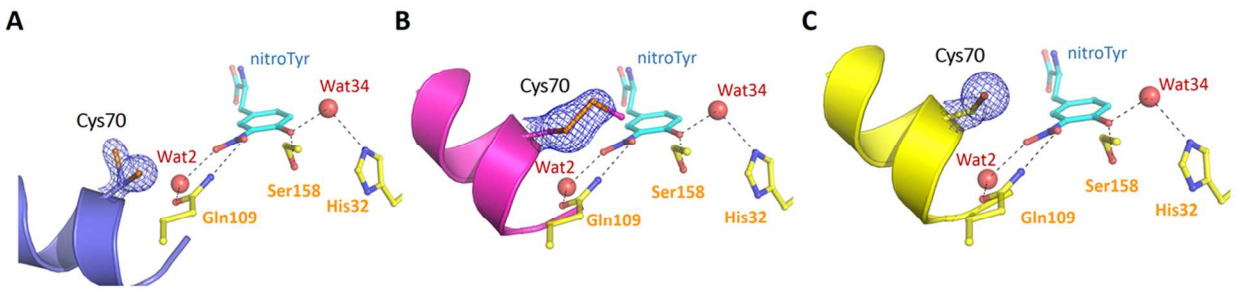
[a] Prepared as previously described by Hammill et al.(*3*)

Supplementary Table 2.

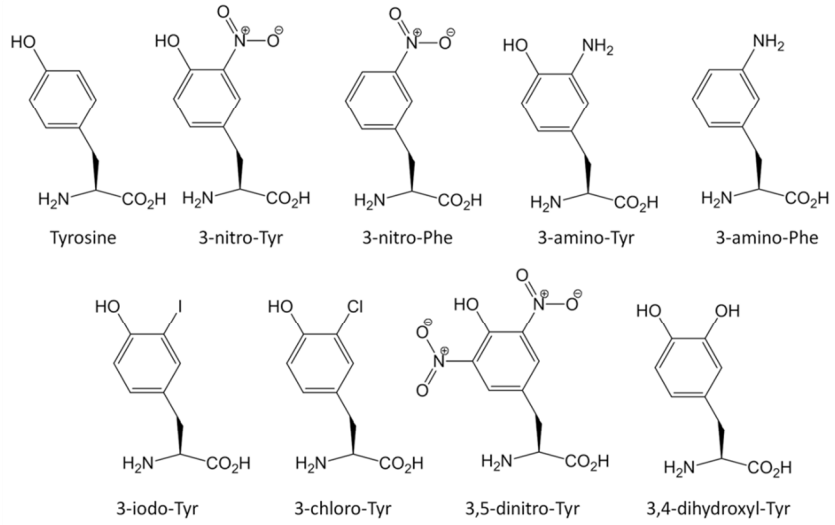
Variant	Residue number								
	32	**65**	**70**	**108**	**109**	**155**	**158**	**159**	**162**
Wild-type	Y	L	H	F	Q	Q	D	I	L
Second generation synthetases									
nitroTyr-5B	H	L	C	F	Q	Q	S	A	R
nitroTyr-1F	L	V	A	R	P	Q	S	L	H
nitroTyr-3B	S	T	L	F	Q	Q	S	A	R
nitroTyr-4C	R	T	G	F	Q	Q	S	E	H
nitroTyr-4E	L	H	G	S	K	Q	S	L	H
nitroTyr-4G	R	T	G	F	Q	Q	G	E	H
nitroTyr-6E	V	V	C	F	Q	Q	S	A	H
nitroTyr-6A	Q	L	L	F	Q	Q	S	P	R
nitroTyr-8B	H	L	A	F	Q	P	S	C	S
nitroTyr-9B	A	T	M	F	Q	Q	S	G	R
nitroTyr-9E	R	L	A	F	Q	Q	S	P	S
nitroTyr-10B	A	T	T	F	Q	Q	S	S	R
First generation synthetases [a]									
nitroTyr-1	E	N	T	F	Q	Q	S	T	L
nitroTyr-4	K	A	M	F	Q	Q	G	M	R
nitroTyr-8	R	L	L	F	Q	M	G	L	H
nitroTyr-10	K	K	S	A	D	Q	G	Y	S
First generation synthetases variants [b]									
nitroTyr-9 (A109F)	V	S	A	F	Q	Q	S	I	L
nitroTyr-2 (L162H)	A	A	G	F	Q	Q	S	A	H
nitroTyr-3 (A108F)	H	A	C	F	Q	P	S	L	R
nitroTyr-5 (S158G)	L	R	H	E	P	Q	G	Y	K
nitroTyr-8 (R32K)	K	L	L	F	Q	M	G	L	H
nitroTyr-8 (L65K)	R	K	L	F	Q	M	G	L	H
nitroTyr-8 (L70A)	R	L	A	F	Q	M	G	L	H
nitroTyr-8 (G158S)	R	L	L	F	Q	M	S	L	H
nitroTyr-2 (A32R)	A	A	G	F	Q	Q	S	A	H
nitroTyr-8 (L70S)	R	L	S	F	Q	M	G	L	H
nitroTyr-8 (L70G)	R	L	G	F	Q	M	G	L	H
nitroTyr-8 (L70A,L65A)	R	A	A	F	Q	M	G	L	H
nitroTyr-8 (L70A,L65S)	R	S	A	F	Q	M	G	L	H
nitroTyr-8 (L70A,L65A)	R	A	A	F	Q	M	G	L	H
nitroTyr-8 (L70A,E36A)	R	A	L	F	Q	M	G	L	H
nitroTyr-8 (L70A,Q109N)	R	A	A	F	N	M	G	L	H
nitroTyr-8 (L70A,E36A, Q109N)	R	A	L	F	N	M	G	L	H

[b]Variants of first generation nitroTyr RSs NitroTyr-8 (L70A), NitroTyr-8 (L70G) and NitroTyr-8 (L70A, L65A) had 1.8 fold, 2.1 fold, and 1.3 fold increase in efficiency, respectively. All others had notable decreases in efficiency.

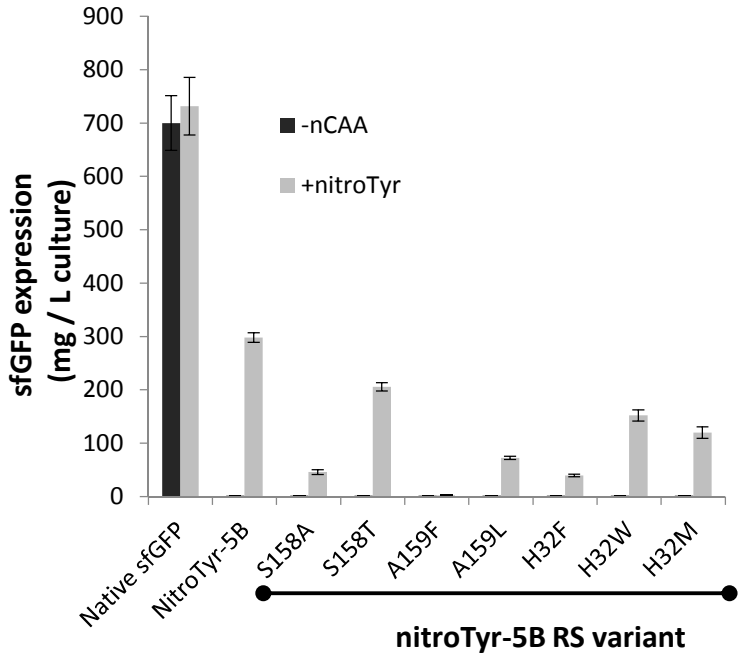
Supplemental Figures.



Supplemental Figure 1. Comparison of Cys70 in the three nitroTyr-5B RS structures. $2F_o$-F_c density for Cys70 contoured at 1.5 ρ_{rms} (blue mesh) for the (A) apo-open, (B) apo-closed and (C) nitroTyr-bound structures of the nitroTyr-5B RS. In the apo-open conformation (panel A), Cys70 adopts two equally populated conformations. Only in the apo-closed structure (panel B) does Cys70 appear to be modified, presumably through disulfide formation with β-mercaptoethanol- a reagent present in the crystallization buffer. The β-mercaptoethanol moiety was disordered such that only the sulfur and its adjoining carbon atom could be modeled. In each panel, the nitroTyr substrate (cyan carbon atoms) and the protein residues with which it interacts (yellow carbon atoms) from the closed-nitroTyr structure are shown in order to depict the relative positions of Cys70 across all three structures. Colors scheme is identical to Figures 2, 3 and 4 of the main text. Hydrogen bonds are indicated by dashed black lines.



Supplemental Figure 2. Structures of tyrosine and non-canonical amino acids related to nitroTyr that were tested for incorporation by the nitroTyr-5B RS.



Supplemental Figure 3. Efficiency and fidelity of nitroTyr-5B RS variants with mutations at the substrate binding pocket. The total yield of sfGFP containing nitroTyr at position 150 expressed in autoinducing cultures is shown in mg protein per liter culture for each nitroTyr-5B RS variant in media lacking (black) and supplemented with (gray) 1 mM nitroTyr. The absolute fidelity appears lower in this experiment compared to that of Figure 1 because the samples were expressed for 30 hrs instead of 40 hrs. It should be noted that both efficiency and absolute fidelity are affected by ncAA concentration, expression length and media composition.

Supplemental References

1. Neumann, H., Hazen, J. L., Weinstein, J., Mehl, R. A., and Chin, J. W. (2008) Genetically encoding protein oxidative damage, *J Am Chem Soc 130*, 4028-4033.
2. Turner, J. M., Graziano, J., Spraggon, G., and Schultz, P. G. (2006) Structural plasticity of an aminoacyl-tRNA synthetase active site, *Proc Natl Acad Sci U S A 103*, 6483-6488.
3. Hammill, J. T., Miyake-Stoner, S., Hazen, J. L., Jackson, J. C., and Mehl, R. A. (2007) Preparation of site-specifically labeled fluorinated proteins for 19F-NMR structural characterization, *Nat Protoc 2*, 2601-2607.