Synthesis of Multi-Protein Complexes through Charge-Directed Sequential Activation of Tyrosine Residues

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Abstract

Site-selective protein-protein coupling has long been a goal of chemical biology research. In recent years, that goal has been realized to varying degrees through a number of techniques, including the use of tyrosinase-based coupling strategies. Early publications utilizing tyrosinase from *Agaricus bisporus* showed the potential to convert tyrosine residues into *ortho*-quinone functional groups, but this enzyme is challenging to produce recombinantly and suffers from some limitations in substrate scope.

Initial screens of several tyrosinase candidates revealed that the tyrosinase from *Bacillus megaterium* (megaTYR) as an enzyme that possesses a broad substrate tolerance. We use the expanded substrate preference as a starting point for protein design experiments and show that single point mutants of megaTYR are capable of activating tyrosine residues in various sequence contexts. We leverage this new tool to enable the construction of protein trimers via a charge-directed sequential activation of tyrosine residues (CDSAT).

Introduction

Site-selective post-translational modification of proteins has long been an object of study for both chemists and biologists. Augmenting the native properties of protein scaffolds to contain fluorophores,^{1,2} drug molecules,³ and other signalling moieties has been of tremendous value to both our understanding biology and the creation of new therapeutics.³⁻⁶ While much work has been done in the attachment of small molecule cargoes to protein scaffolds, the facile attachment of additional whole proteins has proven more difficult. The primary challenge of protein-protein coupling lies in the steric inhibition that must be overcome in joining large molecules, as well as the difficulty in generating suitable selective reaction handles due to the frequent occurrence of most native amino acids. Current methods for the site-selective synthesis of protein conjugates involve the introduction of uniquely reactive unnatural amino acids (UAAs) into the backbone sequence,^{7,8} maleimide-cysteine linkages,⁹ and enzymatic coupling reactions.^{6,10-13}

Although these methods have addressed many important challenges in chemical biology, limitations still exist. The site selective incorporation of unnatural amino acids into proteins relies on stop codon repurposing, which requires special growth media and often lowers protein yield.^{7,8} Thiol-reactive crosslinkers, such as maleimides, are specific to cysteine residues on the protein surface that are rare enough to afford selectivity and can be readily inserted via single base mutagenesis. However, the maleimide reactive handle is challenging to install

on the surface of full proteins.⁹ For many enzymatic coupling techniques, such as sortase,
butelase, and farnesyltransferase, coupling can only occur at the N- and C- termini.^{6,10-13}
Most other coupling approaches such as SpyCatcher/SpyTag require the insertion of bulky
recognition domains.¹⁴

A promising alternative is tyrosinase, a broadly distributed class of enzymes involved
in the melanin synthesis pathway in every kingdom of life. Tyrosinase enzymes normally
catalyze the oxidation of tyrosine to a dopaquinone intermediate, which then cyclizes to
form dopachrome.¹⁵⁻¹⁹ Recent studies have repurposed the commercially available mush-
room tyrosinase (abTYR) to catalyze site specific oxidative coupling reactions at N- and C-
terminal tyrosine residues using different types of coupling partners: strained alkynes,²⁰ and
nucleophilic coupling partners such as secondary amines,²¹ anilines,²² and surface exposed
cysteine residues,²³ which expands the possible sites of enzymatic protein-protein biocon-
jugation past the N- and C- termini. This methodology is versatile, and can take place at
temperatures ranging from 4 °C to room temperature and near-neutral pH, to preserve the
function of sensitive proteins.²³

Despite the recent success and convenience of abTYR, it is not without limitations. In
a recent investigation, abTYR was unable to activate tyrosine residues at the C terminus of
protein L, regardless of linker length.²² Both abTYR and protein L are over 100 kDa in mass,
and are negatively charged, suggesting that electrostatic repulsion and steric hinderance may
be the factors preventing the activation of this particular substrate by abTYR.^{24,25} A smaller,
35.5 kDa tyrosinase from *Bacillus megaterium* (megaTYR, referred to as bmTYR in ref 17),¹⁷
was found to activate C-terminal tyrosines on protein L regardless of linker length. In this
study we characterize the substrate preferences of these tyrosinases, and show that abTYR
is unable to oxidize tyrosines in negatively charged substrates, whereas megaTYR, and the
recently reported tyrosinase from the archaeon, *Candidatus Nitrosopumilus koreensis* (cnk-
TYR),²⁶ are much more promiscuous. In this report, we use this information to generate new
versions of megaTYR selective for tyrosine residues in specific charge contexts. Moreover,

we leverage this new tool to enable the construction of protein trimers via charge-directed sequential activation of tyrosine residues (CDSAT).

Results and discussion

In our search for more sequence permissive enzymes we identified tyrosinases from *Bacillus megaterium* (megaTYR) and *Candidatus Nitrosopumilus koreensis* (cnkTYR) as optimal candidates for recombinant expression.^{26,27} In particular, megaTYR was selected because several previous studies showed that the enzyme tolerated point mutations while retaining high activity to free tyrosine.^{17,27,28} The published crystal structures for megaTYR were beneficial for protein engineering, as they allowed rational design of point mutants and facilitated computational analysis of the protein (Figure 1a).²⁷ In addition, cnkTYR was recently shown to have excellent monophenol monooxygenase activity and to tolerate low temperatures without significant loss in catalytic efficiency.^{19,26}

In addition to the previously published work on protein L,²² the motivation for these explorations was the realization that abTYR was incapable of activating phenols attached to a single stranded DNA (ssDNA) substrate. In contrast, our first experiment with megaTYR showed significant levels of coupling between GFP and the phenol-DNA (Figure S1). This reaction occurs as two distinct steps: the initial activation of the phenol to the *ortho*-quinone followed by addition of the nucleophile. These results suggested that some aspect of the DNA substrate was preventing activation by abTYR but was tolerated by megaTYR (Figure S1). We reasoned that this was likely the high degree of negative charge on the nucleotide substrate.

We next examined the substrate preference of the new tyrosinases, specifically with respect to their charge and steric tolerance. A peptide library with tyrosine residues at their C-termini was obtained, shown in Supplemental Table 1, which contained a variety of charged

87 and neutral sequence elements. The peptides were coupled to Y200C GFP in 30 min reactions
88 and analyzed via ESI-TOF MS to determine conversion to the modified peptide-protein con-
89 jugate. To ensure that peptide coupling was not biased by electrostatic interactions between
90 GFP (pI = 6.2) and the peptides, control reactions were run on a *p*-aminophenylalanine
91 modified MS2 capsid, which has a pI of 8.0. These reactions reached similar levels of mod-
92 ification (Supplemental Figure S2). Comparing abTYR to the non-eukaryotic tyrosinases,
93 it is readily apparent that abTYR is significantly more sensitive to negative charge (Figure
94 1D). In fact, abTYR shows no activity towards any peptide with 4 or more acidic residues
95 adjacent to the terminal tyrosine. Also of note is the fact that megaTYR shows high activ-
96 ity towards all peptide substrates, with a drop in activity only in the case of the RRRRY
97 peptide. These results supported the earlier challenge activating ssDNA substrates using
98 abTYR, and suggest that megaTYR may serve as a highly permissive tyrosine activator.
99 One additional observation of this study was that cnkTyr samples showed no discernable
100 pattern of substrate specificity.

Figure 1: Structure and activity of tyrosinase enzymes from eukaryotic, procaryotic, and archaeal sources. A) Protein structure for abTYR from *Agaricus bisporus* with acidic residues in red and basic residues in blue. PDBID:2Y9X B) Protein structure for megaTYR from *Bacillus megaterium* with acidic residues in red and basic residues in blue. PDBID:6EI4 C) Protein structure for cnkTYR from *Candidatus Nitrosopumilus koreensis* with acidic residues in red and basic residues in blue. Structure was generated using the I-TASSER webserver. D) Peptide reaction screen showing percent conversion of Y200C sfGFP with the indicated peptide. All reactions carried out at 50 μ M Y200C sfGFP, 250 μ M peptide, and 12 U/L equivalent of either abTyR (dark blue), megaTYR (blue), or cnkTyR (light blue) in 50 mM pH 6.5 phosphate buffer for 30 min before quench with 4 mM tropolone and analysis via ESI-TOF-MS. Deconvoluted data were analyzed on www.chartograph.com with percent conversion calculated as percentage of peak area for the modified vs unmodified samples. Asterisks indicate where negligible amounts of protein modification were detected.

selectivities, with cnkTyr represented by a homology model created using the I-TASSER server.,^{25,26,29–32} abTYR is found as a hetero-tetramer and shows significant steric occlusion of the active sites (Figure 1A),²⁵ while megaTYR and cnkTYR are monomeric, and show a much more open active site.^{26,29} Due to their more predictable behaviours and established crystal structures, we focused our work on megaTYR and abTYR in subsequent experiments. However, further exploration or engineering of cnkTyr could yield a more specific phenol monooxygenase capable of acting with higher precision.

In the interest of improving megaTYR for even broader activity we performed an evolutionary conservation analysis of the megaTYR backbone in order to determine target positions for engineering. Using the ConSurf web server, the 100 closest non-redundant homologues of megaTYR were clustered and a multiple sequence alignment was prepared (Figure 2A).^{33–36} A normalized conservation score was calculated for each position using an empirical Bayesian methodology, which accounts for phylogenetic relationships between homologues in its construction of an evolutionary model.³⁷ The evolutionary conservation was mapped onto the megaTYR structure and used to identify mutable anionic residues with low conservation scores (Figure 2B, Table S2). Position R209 was also chosen as a position of interest despite its mild conservation due to its established role in potentiating diphenolase activity, and its proximity to the active site (Figure 2C).²⁷ We also chose to study R280 as a more distal mutable cationic residue with a low conservation score (Table S2). Previously studied mutants at positions V218 and F197 were not further investigated as their impact on megaTYR activity was not beneficial to our purpose of producing a more electrostatically targeted tyrosinase.^{17,28}

130 larger effects on active site potential than more distal residues, and modeled them in a similar
131 manner (Table S3). We found that abTYR monomers had a net potential of -4.4 V due to
132 high numbers of acidic residues on their surfaces, corresponding to an isoelectric point (pI)
133 of only 4.41. When the full active complex of abTYR is considered the net potential drops
134 to -15.7 V.²⁵ In contrast, megaTYR was shown to have a net potential of +4.4 V and a pI
135 of 8.66, correlating well with the observed charge preference.²⁹

136 In accordance with our assumption that more proximal residues would have a larger
137 impact on active site potential, we found that R209H had a potential of +3.0 V, a significant
138 reduction compared to the wild type, whereas R280H has a much smaller impact on the
139 electrostatics near the active site (Figure 3B, Table S2). Similarly, D55K had the largest
140 impact on the active site potential among the D/E to K mutants, corresponding to a potential
141 of +7.33 V. Because of this, we also chose to model D55R to compare the effects of mutating
142 anionic residues to lysine or arginine. While D55K had a potential of +7.33V, D55R showed
143 a slightly more positive net potential of +7.5 V, due to the higher pKa of arginine (Figure
144 3B, Table S2).

Figure 3: Calculations on tyrosinase mutants. A) Surface potential maps of abTYR and several megaTYR mutants with negative charge in red and positive charge in blue. B) Table showing results of charge potential calculations for the various tyrosinase mutants. For each tyrosinase a point charge was placed 14 Å from the copper atoms within the active site and potential charge and pI were calculated using the Epik and MacroModel modules of Maestro developed by Schrödinger. Δ potential is calculated as the change in potential between the wild type and the listed mutant.

Based on data from the conservation screen and potential modelling we generated megaTYR with R209H, D55K, and D55R mutations. After initial screening at a tyrosinase concentration of 5 U/L, it was observed that R209H decreased the charge preference of megaTYR, while D55K and D55R were found to increase the preference for anionic substrates as shown by the higher level of modification with the EEEEEY peptide (Figure 4B, structures with residues highlighted in Figure 4A). To assess the degree of substrate preference we ran competition assays containing the EEEEEY and RRRRY peptides in equal concentrations. In these assays abTYR only showed activity towards RRRRY, while R209H

showed equal preference for the two (Figure 4C). Interestingly, wild type megaTYR showed a 90% preference for EEEEEY, while the D55K mutant showed a 95% preference and D55R a surprising >99% preference for the anionic peptide (Figure 3C). As D55K experienced expression challenges, and was not capable of complete substrate differentiation, we chose to focus on D55R as the higher performing mutant.

We then performed a full substrate screen on each megaTYR variant (wild type, R209H, and D55R) at the same concentration as the initial substrate screen (12 U/L) and found that these mutations only had a significant effect on the enzymatic activity towards highly charged peptide substrates. Interestingly, D55R megaTYR exhibited low conversion for the RRRRY peptide, but retained high levels of activity towards the less highly charged cationic peptides (Figure 4D). Generating sequence orthogonal tyrosinases could enable the facile creation of complex multi-domain proteins by coupling anionic and cationic tagged subunits sequentially. This could be done in a manner similar to peptide synthesis but using entire proteins as building blocks. The controlled production of multi-protein polymers could be an enabling technology for protein based materials, biomedical technologies, and enzyme engineering.

Figure 4: Point mutants of megaTYR and their impact on substrate preference. A) Structural comparison of abTYR vs megaTYR with locations of point mutations highlighted. R209 is shown in yellow, D55 in blue. Structures show acidic (Asp and Glu) residues in red and basic (Lys and Arg) residues in blue. B) Comparison of reactivity of abTYR vs single mutants of megaTYR on peptides EEEEEY and RRRRY. Reactions were run for 30 min at RT in 50 mM pH 6.5 phosphate buffer, 5 U/L tyrosinase, 50 μ M Y200C sfGFP and 250 μ M peptide. Reactions were quenched with 4 mM tropolone. C) Competition reactions between RRRRY and EEEEEY peptides to assess substrate preference of tyrosinase mutants. Reactions were run for 30 min at RT in 50 mM pH 6.5 phosphate buffer, 5 U/L tyrosinase, 50 μ M Y200C sfGFP and 250 μ M of each peptide. Reactions were quenched with 4 mM tropolone. D) Peptide reaction screen showing percent conversion of Y200C sfGFP with the indicated peptide. All reactions were run at 50 μ M Y200C sfGFP, 250 μ M peptide, and 12 U/L equivalent of either wt megaTYR (blue), R209H megaTYR (dark blue), or D55R megaTYR (light blue) in 50 mM pH 6.5 phosphate buffer for 30 min before quenching with 4 mM tropolone and analysis via ESI-TOF-MS. Deconvoluted data were analyzed on www.chartograph.com with percent conversions calculated as percentage of peak area percentages for the modified vs unmodified species.

Although the megaTYR mutants to date were not fully prevented from acting on cationic substrates, the range in selectivity between abTYR and D55R megaTYR proved a tantalizing target. To test this, the cysteine-containing Y200C sfGFP was modified with EEEEEY and RRRRY C-terminal tags, which should be unreactive with abTYR, and D55R megaTYR, respectively (Figure S3, S4). After purification, the Y200C sfGFP-EEEEY (GFP-E4Y) was exposed to abTYR for 30 min in a variety of conditions. Notably, the mushroom tyrosinase was able to append GGGGY and RRRRY peptides to the GFP-E4Y without oxidizing the EEEEEY tag. In addition, exposing the GFP-E4Y to abTYR resulted in no discernable oxidation by ESI-MS. This aligns with our previous peptide screens, and suggests that the EEEEEY tag is not susceptible to abTYR activation (Figure S3). Similarly, Y200C sfGFP-RRRRY (GFP-R4Y) was exposed to D55R megaTYR under similar conditions, with the EEEEEY peptide used in place of the RRRRY peptide. Similarly to abTYR, D55R megaTYR was able to selectively oxidize the added peptides without modifying the tyrosine tag on the GFP-R4Y. Reaction selectivity was further confirmed when exposure of GFP-R4Y to D55R megaTYR alone did not result in any oxidation (Figure S4). Together these data suggest that abTYR and D55R megaTYR can be used for CDSAT on proteins and peptides.

To further validate reaction orthogonality we next exposed the GFP-E4Y to a phenol-biotin in the presence of abTYR and showed selective oxidation of the small molecule to afford the GFP-E4Y-biotin conjugate (Figure 5E). In parallel, we modified GFP-E4Y with a biotin-maleimide as a positive control (Figure 5D). After purification using a 10 kDa MWCO spin concentrator, both the maleimide and phenol GFP-E4Y-biotin were combined with Y200C sfGFP and 12 U/L D55R megaTYR for 30 min and analyzed via ESI-TOF-MS. We observed over 70% conversion to the trimer composed of two GFP proteins and a biotin molecule, making this one of the first techniques capable of creating 3-component fusions without the need for solid support tethering or removable protective groups (Figure 5F-G). In addition, some higher order modifications appeared, arising from residual disulfide formation between unreacted thiols in the first step.

Figure 5: Coupling of two proteins to a small molecule. A) Scheme depicting the construction of a cysteine GFP (Y200C GFP), as well as a negative tyrosine tagged cysteine GFP (GFP E4Y). B,C) ESI-MS data for unmodified Y200C GFP and GFP E4Y respectively. D) ESI-MS of GFP E4Y modified with biotin maleimide. Biotin-maleimide coupled to GFP E4Y by combining 50 μ M GFP E4Y with 500 μ M biotin-maleimide at pH 7.5 for 2 h. E) GFP E4Y was coupled to biotin-phenol by combining 50 μ M GFP E4Y with 200 μ M biotin-phenol with 12 U/L abTYR in 50 mM phosphate pH 6.5 for 30 min at RT. Product mass expected = 29,054 Da. F-G) Reaction of 10 μ M GFP-E4Y-biotin with 50 μ M Y200C GFP with 12 U/L megaTYR for 30 min at RT. Expected mass for GFP+GFP+ maleimide biotin = 56,446 Da and for GFP+GFP phenol = 56,618 Da.

To evaluate the potential of an iterative tyrosinase sequence for the creation of protein trimers, GFP-E4Y was combined with a MYGGS-tagged nano-luciferase and 12 U/L ab-TYR to activate the neutral luciferase tag selectively (Figure 6A-C). Clean conversion to the GFP-E4Y-nano-luciferase dimer was observed, with no excess oxidation. To remove any

unreacted starting material, the solution was purified using a 50 kDa MWCO spin concentrator (Figure 6C). As a third component, the thiol-containing S152C-mCherry was added to the dimer solution with 12 U/L D55R megaTYR to activate the EEEEEY tag on the GFP. This strategy led to smooth conversion to the mCherry-GFP-luciferase trimer (Figure 6D). We saw 100% conversion from the dimer to the final product, indicating that megaTYR is capable of catalyzing tyrosinase oxidation on anionic tags even in the presence of multi-protein complexes. The tri-functional nature of the construct identity was further validated by luminescence and fluorescence measurements. Upon introducing the nanoLuc substrate, FRET was observed between the luciferase, GFP, and mCherry subunits. In addition, direct excitation of the GFP fluorophore led to emission from the mCherry segment via energy transfer (Figure S5). This new capability to oxidize tyrosine residues in different charge contexts iteratively highlights the potential of this system to generate complex protein products for future applications.

Figure 6: Use of tyrosinase charge selectivity for the synthesis of ternary protein complexes. A) Reaction scheme showing the use of sequential reactions with abTYR and megaTYR to couple 3 proteins together. B) Protein sequence maps for S152C mCherry (mCherry), Y200C GFP with EEEEEY tag (GFP E4Y), and a MYGGS tagged nano Luciferase (nanoLuc). C) Reaction of 50 μ M GFP-E4Y with 100 μ M nanoLuc and 12 U/L abTYR for 30 min. GFP + nanoLuc structure shown above chart. Expected mass = 48,728 D) GFP E4Y-nanoLuc (10 μ M) purified by spin down using a 50 kDa MWCO spin concentrator combined with 100 μ M Y200C GFP and 12 U/L megaTYR for 30 min at RT converted E4Y GFP-nanoLuc to the mCherry-GFP-nanoLuc trimer after purification using 50 kDa MWCO spin filter. Trimer structure shown above spectrum. Expected mass = 76,153 Da.

Conclusion

Here we show that recombinantly expressed tyrosinases are successful activators of terminal tyrosine residues on a variety of protein and peptide substrates. The results from the megaTYR mutant screen show that bacterially derived tyrosinases possess sufficient selectivity to mediate protein-protein coupling reactions through tyrosine-cysteine bond formation, and can be engineered to alter their substrate preferences. The success at modulating the charge preference of this enzyme indicates that it is tolerant toward mutation and can be engineered to achieve orthogonal reactivity preferences to those of abTYR. The result of these efforts is the ability to sequentially activate tyrosine residues based on their charge context, or CDSAT. The protein trimers created using CDSAT show that minimal engineering is required to produce a system capable of creating higher-order protein constructs. Such multivalent conjugates have tremendous potential for materials, therapeutic, and diagnostic applications.

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Supporting Information Available

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Water (ddH₂O) used in biological procedures or as a reaction solvent was de-ionized using a NANOpure purification system (Barnstead, USA). Peptides were purchased from GenScript (Piscataway, NJ). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Terrific broth used in protein expression was purchased from Mediatech Inc. (Maryland, VA). Spin concentrators were obtained from EMD Millipore (Millipore, Billerica, MA).

Instruments and Characterization

Mass Spectrometry. Time of flight electrospray ionization mass spectrometry (TOF-ESI-MS) of proteins was performed using an Agilent 1260 series liquid chromatography (LC) system with an Agilent 6530C Q-TOF LC-MS system (Santa Clara, CA). For mobile phases, the system utilized Milli-Q H₂O with 0.1% (v/v) formic acid (solvent A) and HPLC grade ACN with 0.1% (v/v) formic acid (solvent B). Before submission to analysis, each sample was desalted and passed through a 0.22 μ m cellulose acetate centrifugal filter. Samples of 2-10 μ L were injected, corresponding to 0.2 to 1.0 μ g of protein. The sample was separated on a Proswift RP-4H (monolithic phenyl, 1.0 mm $\times$ 50 mm, Dionex) analytical column with a flow rate of 0.30 mL/min and a gradient of 5-100% solvent B in solvent A over 8 min. Agilent Mass Hunter Workstation software (Qualitative Analysis Version B.10.0, Build

10.0, Agilent Technologies Inc.© 2020) was used to extract total ion chromatograms from raw data and the Maximum Entropy deconvolution algorithm was used to reconstruct the original analyte masses. CSV files of spectra were plotted using the open source Chartograph software (www.chartograph.com). Integration of an unmodified protein sample served as an internal standard for determining conversion.

Size Exclusion HPLC:

HPLC purification of Encapsulated proteins was performed on an Agilent 1100 Series HPLC Systems (Agilent Technologies, USA) outfitted with an Agilent 1200 series automatic fraction collector. Sample analysis for all HPLC experiments was achieved with an inline diode array detector (DAD) and in-line fluorescence detector (FLD). HPLC of capsids and proteins was accomplished using a BioSep-SEC s4000 column 300 x 7.8 mm column (Phenomenex, Torrance, CA). The samples were eluted in isocratic flow of 10 mM or 100 mM pH 6.8 phosphate buffer at 1 mL/min.

UV-Vis Spectroscopic Measurements:

UV-Vis spectroscopic measurements were conducted on a Thermo Scientific nanoDrop 1000 scan benchtop spectrophotometer (Thermo Scientific, Wilmington, DE). Spectra were collected in Proteins and Labels mode from 200-800nm, while direct protein concentrations were calculated using Protein A280.

Gel Analyses:

For protein analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a NuPAGE apparatus (Life Technologies, Hercules, CA), using a 4-12% precast linear gradient polyacrylamide gel (Bio-Rad, Hercules, CA). All protein electrophoresis samples were heated for 10 min at 75 °C in the presence of 1,4-dithiothreitol (DTT) to ensure reduction of disulfide bonds. Gels were run for 30 min at 200 V to separate the bands. Commercially available markers (Bio-Rad) were applied to at least one lane of each gel for assignment of apparent molecular masses. Visualization of protein bands was accomplished by staining with Coomassie Brilliant Blue R-250 (Bio-Rad). Gels were

visualized on Gel-Doc EZ Imager (Bio-Rad).

For PCR reactions products were run on a 2% agarose gel in TEA buffer with 1:10000 SyberSafe DNA gel stain (ThermoFisher Scientific, Wilmington, DE). PCR samples were incubated for 5 min with 6x Orange loading buffer (Orange Loading Dye, Thermo Scientific, Waltham, MA) before application to the gel. Gels were run for 45 min at 120 V to separate bands. Commercially available markers (1kb DNA Ladder, New England BioLabs, Ipswich, MA) were applied to at least one lane of each gel for assignment of apparent mass. Visualization of DNA bands was accomplished using a Gel-Doc EZ Imager (Bio-Rad).

Fluorimeter Measurements:

Fluorimeter measurements were conducted on a Fluoromax-4 Spectrofluorometer (HORIBA Scientific). Proteins were placed in a cuvette with 1 mL of 50 mM pH 6.5 phosphate with constant stirring using magnetic stir bar. Final protein concentration for all experiments was 250 nM which was obtained by diluting 5 μ L of 50 μ M stock in 995 μ L 50 mM pH 6.5 phosphate. For nanoLuciferase assay, 5 μ L of NanoLuc Substrate (Promega) was diluted in 95 μ L of NanoLuc Buffer (Promega), then added in 5 μ L aliquots to the prepared sample already inserted into the Fluorimeter. Measurements were taken after addition as quickly as possible measuring from 400 nm to 700 nm at 5 nm increments. For direct fluorescence measurements readings were taken by exciting at 485 nm with a 5 nm window and monitoring emission from 510-700 nm for GFP. For mCherry detection readings were taken by exciting at 585 nm with a 5 nm window and monitoring emission from 610-700 nm.

Protein Expression and Purification:

Expression and purification of prokaryotic tyrosinases:

A 10 mL overnight culture of BL21 (DE3) Codon Plus RIL *E. coli* in Terrific Broth (TB) medium with 50 μ g/mL kanamycin was inoculated into a 2.8 L trident Erlenmeyer flask with 1 L of sterile TB with kanamycin. The culture was incubated at 37 °C, 220 rpm and cell density was monitored until the O.D. at 600 nm was between 0.4 and 0.6. The cells were then induced by adding IPTG to a final concentration of 0.4 mM and subsequently

incubated for another 12 h at 37 °C. The cells were then pelleted and stored at -80 °C.

The cell pellet was thawed on ice in lysis buffer (500 mM NaCl, 20 mM imidazole, 20 mM Tris-HCl pH 7.5) and PMSF (0.1 mM), and cells were lysed by sonication for 10 min (2 s on, 4 s off, 60% amplitude, with a Fisher Scientific Sonic Dismembrator). The cellular debris was removed with centrifugation (17,000 g, 15 min, 4 °C), and the supernatant was applied to a Ni-NTA gel spin column (ThermoFisher HisPur). The column was washed 3 times with 6 mL of lysis buffer (lysis buffer without PMSF), and fractions were collected. The protein was then eluted using four 6 mL portions of elution buffer (500 mM NaCl, 500 mM imidazole, 20 mM Tris-HCl, 0.02 mM CuSO₄, pH 7.5) for a total of 24 mL. All fractions were analyzed using SDS-PAGE, and the tyrosinase-containing fractions were collected. The buffer was exchanged for protein storage buffer (PBS with 15% glycerol and 0.02 mM CuSO₄) using a spin concentrator (10 KDa MWCO, 15 mL, Amicon Ultra) and then concentrated to 150 μ M. The tyrosinase solutions were stored at -80 °C.

Expression and purification of Y200C-sfGFP variants:

After sequence verification, a 10 mL overnight culture of BL21 (DE3) Codon Plus RIL *E. coli* in TB medium with 50 μ g/mL kanamycin was inoculated into a 2.8 L trident Erlenmeyer flask with 1 L of sterile Terrific Broth (TB) medium and kanamycin. The cultures were grown at 37 °C with 220 rpm shaking until O.D. at 600 nm reached 0.6 – 0.8. To induce expression, IPTG was added from a 1.0 M stock to a final concentration of 1.0 mM. The cultures were incubated at 37 °C for 12-16 h. Cells were then incubated for another 12-16 h at 37 °C and pelleted. After freezing at -80 °C, cell pellets were resuspended in 15 mL of an equilibration buffer (20 mM sodium phosphate, 2 M NaCl, 20 mM imidazole at pH = 7.4) and then lysed via sonication for 30 min at 60% amplitude. Cell lysate was spun down at 14,000 rpm for 30 min, then the supernatant decanted before loading on to a HisTrap FF Crude column. After binding, the bound protein was washed with 4 portions of two resin bed volumes of wash buffer (20 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole at pH = 7.4) and subsequently eluted with four resin bed volumes of elution buffer (20 mM sodium phosphate,

300 mM NaCl, 250 mM imidazole at pH = 7.4). The purified sfGFP variant was then spun concentrated into 50 mM phosphate buffer at pH 7.2 using 10k MWCO filters. Purified sfGFP samples were flash frozen and stored at -20 °C until use. Purified protein was spin concentrated into 20 mM phosphate buffer at pH 7.0 using 10k MWCO spin filters prior to reaction.

Expression and purification of paF MS2:

Glycerol stocks of *E. coli* containing plasmids for the T19paF mutant of MS2 were generously provided by Ioana Aanei. The T19paF MS2 expression was carried out in minimal media following the published protocol.³⁸⁻⁴⁰ The pellets were thawed and re-suspended in 20 ml of 20 mM taurine buffer (pH 9) containing 6.5 mM DTT, 6 mM MgCl₂, and 10 µg/ml each of DNase and RNase. Following sonication for 10 min, the cells were spun down for 45 min at 11,000 rpm. Next, the supernatant was applied to a DEAE-Sephadex column (GE Healthcare, Little Chalfont, United Kingdom). In 20 mM pH 9 taurine buffer, MS2 eluted first from the DEAE column and was collected and precipitated using an equal volume of saturated aqueous ammonium sulfate. The protein pellets were re-suspended in 10 mM pH 7.2 phosphate buffer and applied to a Sephacryl S1000 column (GE Healthcare). The fractions containing MS2 were then collected and concentrated using Amicon Ultra 100 kDa MWCO centrifugal concentrators (Millipore). A yield of 3 mg/L culture was obtained for T19paF MS2 following two rounds of purification.

MS2 paF sequence:

T in red represents the T19paF mutation site:

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1  ASNFTQFVLV DNGGTGDVTV APSNFANGVA EWISSNSRSQ AYKVTCSVRQ SSAQNRKYTI
61 KVEVPKVATQ TVGGVELPVA AWRSYLN MEL TIPIFATNSD CELIVKAMQG LLKDGNPPIPS
121 AIAANS GIY

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Expression of nano luciferase (nanoLuc):

The gene encoding nanoLuc was cloned from plasmids provided by Promega (pNL1.1)

using primers that encoded the specified N- or C-terminal modifications as well as a golden gate cloning site. The modified product was then transferred into a modified pet28b cloning vector (pECH081) and transformed into Rosetta DE3 pLysS E. coli. Overnight cultures were grown at 37 °C in LB with 50 mM kanamycin, then transferred into 1 L TB media with 50 mM kanamycin. Once cultures reached an OD600 of 0.6 protein production was induced by addition of 100 mg/mL IPTG and left shaking overnight at 37 °C. After 16 h of incubation, the cultures were centrifuged at 7000 x g, and the pellets collected were then re-suspended in 50 mM phosphate, 200 mM NaCl, 20 mM imidazole, pH 7 buffer. Samples were lysed by sonication, then centrifuged at 17000 x g. The supernatant was collected and run through a HiTrap NiNTA column from GE in a miniAkta, and eluted with a 50 mM phosphate, 200 mM NaCl, 250 mM imidazole buffer at pH 7. Purified nanoLuc samples were flash frozen and stored at -20 °C until use. Purified protein was spin concentrated into 20 mM phosphate buffer at pH 7 using 10k MWCO spin filters prior to reaction.

Protein Sequences and DNA Manipulation:

Protein gene blocks were ordered from Integrated DNA Technologies, codon-optimized for E. coli expression. BsaI cut sites were present at either end of the gene sequence for cloning into the pET28b golden gate entry.⁴¹ This vector enabled green / white screening for colonies successfully transformed with plasmids bearing the inserted gene and provided resistance to kanamycin. PCR primers used to make megaTYR and sf-GFP variants are listed in Supporting Table 2.

Mutagenesis was performed using a combination of Gibson Assembly and Golden Gate based approaches. In the Gibson Assembly based approach, two sets of primers were used to create two linear half plasmids bearing the R209H mutation, which were then cyclized using a standard Gibson Assembly protocol. In the Golden Gate approach, two primers were used to create a D55K and D55R linearized plasmids flanked by BsaI cutsites, which was then cyclized using a standard Golden Gate Assembly protocol.

DNA and Protein Sequences:

385 *Y200C-sfGFP*-His6 Protein sequence:

1 MRKGEELFTG VVPILVELDG DVNGHKFSVR GEGEGDATNG KLTLKFICTT GKLPVPWPTL
61 VTTLTYGVQC FARYPDHMKQ HDEFFKSAMPE GYVQERTISF KDDGTYKTRA EVKFEGDTLV
386 121 NRIELKGIDF KEDGNILGHK LEYNFNHSHV YITADKQKNG IKANFKIRHN VEDGSVQLAD
181 HYQQNTPIGD GPVLLPDNHC LSTQSVLSKD PNEKRDHMLV LEFVTAAGIT HGMDLYKHH
387 241 HHHH*

388 Y200C sfGFP was expressed as previously reported^{21,23}

389 Y200C-sfGFP-His6-RRRRY Protein sequence:

1 MRKGEELFTG VVPILVELDG DVNGHKFSVR GEGEGDATNG KLTLKFICTT GKLPVPWPTL
61 VTTLTYGVQC FARYPDHMKQ HDEFFKSAMPE GYVQERTISF KDDGTYKTRA EVKFEGDTLV
390 121 NRIELKGIDF KEDGNILGHK LEYNFNHSHV YITADKQKNG IKANFKIRHN VEDGSVQLAD
181 HYQQNTPIGD GPVLLPDNHC LSTQSVLSKD PNEKRDHMLV LEFVTAAGIT HGMDLYKHH
391 241 HHHHRRRRY

392 Y200C-sfGFP-His6-EEEEY Protein sequence:

1 MRKGEELFTG VVPILVELDG DVNGHKFSVR GEGEGDATNG KLTLKFICTT GKLPVPWPTL
61 VTTLTYGVQC FARYPDHMKQ HDEFFKSAMPE GYVQERTISF KDDGTYKTRA EVKFEGDTLV
393 121 NRIELKGIDF KEDGNILGHK LEYNFNHSHV YITADKQKNG IKANFKIRHN VEDGSVQLAD
181 HYQQNTPIGD GPVLLPDNHC LSTQSVLSKD PNEKRDHMLV LEFVTAAGIT HGMDLYKHH
394 241 HHHHGGSEEE EY*

1 ATGTCGAATA AATATCGTGT GCGTAAGAAC GTCTTGCATC TGACGGACAC AGAGAAACGC
61 GACTTTGTTC GCACCGTTCT TATCTTAAAA GAAAAAGGGA TTTATGATCG CTACATCGCA
121 TGGCACGGAG CCGCAGGCAA GTTCCACACG CCGCCTGGAA GCGATCGCAA TGCGGCGCAC
181 ATGTCGTCAG CTTTCTTACC ATGGCACCGC GAATATTTAT TGCGTTTTGA CGCGACCTTC
241 AAAGCATTA A CCCCAGAGTC AACTGCCCT ATTGGGAGTG GGAAACCGAT GCTCAAATGC
301 AAGATCCTCT CAATCTCAGA TTTGGTCCGC AGACTTTATG GGGGAAACG GTAATCCGAT
361 CAAGGATTTT ATCGTGGACA CGGGACCATT TGCGGCAGGG CGCTGGACCA CGATTGATGA
396 421 GCAAGGAAAC CCTAGTGGGG GTCTGAAACG TAATTTCCGT GCGACAAAAG AAGCGCCCAC
481 ATTGCCTACT CGTGACGATG TCTTGAACGC ACTTAAAATC ACGCAGTATG ACACGCCGCC
541 GTGGGATATG ACTTCGCAGA ACTCTTTCCG CAATCAGCTG GAAGGCTTTA TTAACGGGCC
601 TCAGCTTCAC AATCGCGTCC ACCGCTGGGT AGGCGGACAA ATGGGTGTTG TGCCCACTGC
661 CCCGAATGAC CCCGTCTTCT TCTTGCATCA TGCTAACGTC GACCGTATCT GGGCCGTCTG
721 GCAGATCATC CACCGCAACC AGAATTACCA ACCGATGAAG AACGGCCCAT TCGGGCAGAA
781 CTTCCGCGAT CCTATGTACC CCTGGAATAC AACACCCGAA GACGTTATGA ATCATCGCAA
841 ATTAGGTTAC GTTTATGACA TCGAACTTCG CAAGAGTAAG CGCTCCTCTc tcgagcacca
397 901 ccaccaccac cactga

398 Corresponding to:

1 MSNKYRVRKN VLHLDTEKR DFVRTVLILK EKGIYDRYIA WHGAAGKFHT PPGSDRNAAH
61 MSSAFLPWHR EYLLRFERDL QSINPEVTLP YWEWETDAQM QDPSQSQIWS ADFMGGNGNP
399 121 IKDFIVDTGP FAAGRWTID EQGNPSGGLK RNFGATKEAP TLPTRDDVLN ALKITQYDTP
181 PWDMTSQNSF RNQLEGFING PQLHNRVHRW VGGQMGVVPT APNDPVFFLH HANVDRIWAV
241 WQIIHRNQNY QPMKNGPFGQ NFRDPMYPWN TTPEDVMNHR KLGYYVDIEL RSKSRSSLEH
400 301 HHHHH*

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Candidatus Nitrosopumilus koreensis Tyrosinase-His6

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1 ATGACCCGTA AAAACGCTAA AGATTTCTTT CCCGACGAGC GCCAACGCTA CTGTAACGCT
 61 TTGCTGACAC TGAAGCAGAC GATCGAACCT GGGCACACGT TGAGCAAGTA TGACGAATTT
 121 GTAGCTATCC ACTACGGTGT CACCCGCCGC TTACGCAACG GCATTCCAAT CGGAGATGGA
 181 GCCCACTTCG TGCCGGGCTT TCTTGCTTGG CATCGTGAGT ATCTGAATCG CTTTGAGAAG
 241 GCAATTCGCA CAGTGGACAG TACCTTATCA CTGCCTTACT GGAATTGGTC TAGTGGTGAT
 301 GATACAGATA CGACCGAGAT CTTACAGAC GATTTTATGG GACCCCGGG AGATCCGAAC
 361 AATGGCAACA AAATTACATC CGGCTATTTT GTAGAGAATA ACTGGGAGGT TCACTCTGAG
 421 CTGGACGGCG GGAACAATGG TTCGGTTCTT GTCCGTGACT CCACACTGTT ATCTTCATCA
 481 AAGTTATCTC AGGTCTCGGG TTACGGCGAA TTGGCTATGG ATGCCGTAAA TGGTGATAAC
 541 GACTTCGACT CATTCTGCC TGGCTTAGAG GGTCCACATG GTTCCATCCA CATGTGGATC
 601 GGTGGACATA TGACCAGTAT GACCAGTCCC AACGATCCTA TTTTCTTCCT TCATCACGCC
 661 AATATTGATC GTTTGTGGAG TAAGTGGCAA GAGTTGCATC CTGGGCCGGA GAACTATAAT
 721 CCAAACAACG ACGGTTTATA TGGCAGTCGC CTTAATGACC GTATGTGGCC GTGGGACGGC
 781 TCCGAGGACA CCACAACACT ACGCACCAGA ACCGCAACAG GGATCTCTCT GCAAGGTTTA
 841 CTTCTACCT TTAGTGATTA TGATATTGTA ACACCGCGCC ATGTGCTTGA TAACAATTTG
 901 ACGATTCCGC ACCACCATCA CCACCATTAA

Corresponding to:

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409

1 MTRKNAKDFL PDERQRYCNA LLTLKQTIEP GHTLSKYDEF VAIHYGVTRR LRNGIPIGDG
 61 AHFVPGFLAW HREYLNRFK AIRTV DSTLS LPYWNWSSGD DTDTEIFTD DFMGPPGDPN
 121 NGNKITSGYF VENNWEVHSE LDGGNNGSVL VRDSTLLSSS KLSQVSGYGE LAMDAVNGDN
 181 DFDSFLPGLG GPHGSIHMI GGHMTSMTSP NDPIFFLHHA NIDRLWSKWQ ELHPGPENYN
 241 PNNDGSYGSR LNDRMWPWDG SEDTTTTRTG TATGISLQGL LPTFSDYDIV TPRHVLNNDL
 301 TIPHHHHHH*

410

MYGGS His6 NanoLuciferase

411

1 ATGTATGGTG GTTCACACCA TCACCATcac catGTCTTCA CACTCGAAGA TTTCGTTGGG
 61 GACTGGCGAC AGACAGCCGG CTACAACCTG GACCAAGTCC TTGAACAGGG AGGTGTGTCC
 121 AGTTTGTTC AGAATCTCGG GGTGTCCGTA ACTCCGATCC AAAGGATTGT CCTGAGCGGT
 181 GAAAATGGGC TGAAGATCGA CATCCATGTC ATCATCCCGT ATGAAGGTCT GAGCGGCGAC
 241 CAAATGGGCC AGATCGAAAA AATTTTAAAG GTGGTGTACC CTGTGGATGA TCATCACTTT
 301 AAGGTGATCC TGCACTATGG CACACTGGTA ATCGACGGGG TTACGCCGAA CATGATCGAC
 361 TATTTCCGAC GGCCGTATGA AGGCATCGCC GTGTTCGACG GCAAAAAGAT CACTGTAACA
 421 GGGACCTGT GGAACGGCAA CAAAATTATC GACGAGCGCC TGATCAACCC CGACGGCTCC
 481 CTGCTGTTCC GAGTAACCAT CAACGGAGTG ACCGGCTGGC GGCTGTGCGA ACGCATTCTG
 541 GCGTAA

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413

414

Corresponding to:

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1 MYGGSHHHHH HVFTLEDFVG DWRQTAGYNL DQVLEQGGVS SLFQNLGVSV TPIQRIVLSG
 61 ENGLKIDIHV IIPYEGLSGD QMGQIEKIFK VVYPVDDHHF KVILHYGTLV IDGVTPNMID
 121 YFGRPYEGIA VFDGKKITVT GTLWNGNKII DERLINPDGS LLFRVTINGV TGWRLCERIL
 181 A*

416

417

Figure S1: SDS-PAGE Gel analysis of DNA-GFP constructs. A) SDS-PAGE Gel analysis of tyrosinase activity towards phenol-DNA. B) Reaction scheme indicating the tyrosinase mediated coupling of phenol-DNA to Y200C GFP

Figure S2: paF-MS2-peptide screen of abTYR substrate preferences. 50 μ M paF-MS2, 250 μ M peptide, 12 U/L abTYR, 30 min at room temperature.

Figure S3: Selective activation of tyrosines on proteins and peptides with abTYR. A-C) Reaction schemes depicting the reaction of EEEEEY Y200C GFP with GGGGY and RRRRY peptides, along with a control reaction without peptide. D-E) abTYR (12 U/L) catalyzed reactions of EEEEEY Y200C GFP with 250 μ M GGGGY or RRRRY peptide. F) EEEEEY Y200C GFP negative control. G-H) abTYR (12 U/L) catalyzed reactions of EEEEEY Y200C GFP with 150 μ M GGGGY or RRRRY peptide. I) EEEEEY Y200C GFP exposed to 12 U/L of abTYR for 30 min in the absence of peptide.

Figure S4: Selective activation of tyrosines on proteins and peptides with D55R megaTYR. A-C) Reaction schemes depicting the reaction of RRRRY Y200C GFP with GGGGY and EEEEEY peptides, along with a control reaction without peptide. D-E) D55R megaTYR (12 U/L) catalyzed reactions of RRRRY Y200C GFP with 250 μ M GGGGY or EEEEEY peptide. F) RRRRY Y200C GFP negative control. G-H) D55R megaTYR (12 U/L) catalyzed reactions of RRRRY Y200C GFP with 150 μ M GGGGY or EEEEEY peptide. I) RRRRY Y200C GFP exposed to 12 U/L of D55R megaTYR for 30 min in the absence of peptide.

Figure S5: Supplemental data for figure 6. A-C) MYGGS nano-luciferase, EEEEEY Y200C GFP, and S152C mCherry negative controls D-E) Dimeric and trimeric protein constructs made by taking advantage of native and engineered charge preferences of tyrosinases F) Fluorescence analysis confirming the proximity FRET triplet of luciferase, GFP and mCherry.

Representative Spectra
Figure 1D

Figure S6: Representative spectra for figure 1D A-C) Deconvoluted and raw mass spectra for the product of the abTYR catalyzed reaction of Y200C GFP and NNNNY peptide D-F) Deconvoluted and raw mass spectra for the product of the wild type megaTYR catalyzed reaction of Y200C GFP and NNNNY peptide. G-I) Deconvoluted and raw mass spectra for the product of the cnkTYR catalyzed reaction of Y200C GFP and NNNNY peptide.

Representative Spectra
Figure 4B and 4C

Figure S7: Representative spectra for figure 4B and 4C A-C) Deconvoluted and raw mass spectra for the product of the megaTYR catalyzed reaction of Y200C GFP and RRRRY peptide D-F) Deconvoluted and raw mass spectra for the product of the D55R megaTYR catalyzed reaction of Y200C GFP and EEEEEY peptide. G-I) Deconvoluted and raw mass spectra for D55R megaTYR EEEEEY vs. RRRRY competition assay.

Representative Spectra Figure 4D

Figure S8: Representative spectra for figure 4D A-C) Deconvoluted and raw mass spectra for the product of the wild type megaTYR catalyzed reaction of Y200C GFP and EEEEEY peptide D-F) Deconvoluted and raw mass spectra for the product of the D55R megaTYR catalyzed reaction of Y200C GFP and EEEEEY peptide. G-I) Deconvoluted and raw mass spectra for the product of the R209H megaTYR catalyzed reaction of Y200C GFP and EEEEEY peptide.

Representative Spectra
Figure 5

Figure S9: Representative spectra for figure 5 A-C) Deconvoluted and raw mass spectra for the product of the abTYR catalyzed reaction of EEEEEY Y200C GFP and phenol-biotin D-F) Deconvoluted and raw mass spectra for the product of the D55R megaTYR catalyzed reaction of maleimide-biotin EEEEEY-GFP and Y200C GFP peptide. G-I) Deconvoluted and raw mass spectra for the product of the D55R megaTYR catalyzed reaction of phenol-biotin EEEEEY-GFP and Y200C GFP.

Figure S10: Representative spectra for figure 6 A-C) Deconvoluted and raw mass spectra for the S152C mCherry control D-F) Deconvoluted and raw mass spectra for the product of the abTYR catalyzed reaction of EEEEEY Y200C GFP and MYGGS nano luciferase. G-I) Deconvoluted and raw mass spectra for the product of the D55R megaTYR catalyzed reaction of the luciferase-GFP-EEEEY coupling product and S152C mCherry.

Representative Spectra Figure S3

Figure S11: Representative spectra for figure S3 A-C) Deconvoluted and raw mass spectra for the product abTYR catalyzed reaction between EEEEY Y200C GFP and RRRRY peptide D-F) Deconvoluted and raw mass spectra for the product of the abTYR catalyzed reaction of EEEEY Y200C GFP and GGGGY peptide. G-I) Deconvoluted and raw mass spectra for the product of the reaction between abTYR and EEEEY Y200C GFP.

Representative Spectra
Figure S4

Figure S12: Representative spectra for figure S4 A-C) Deconvoluted and raw mass spectra for the product D55R megaTYR catalyzed reaction between RRRRY Y200C GFP and EEEEEY peptide D-F) Deconvoluted and raw mass spectra for the product of the reaction between D55R megaTYR and RRRRY Y200C GFP. G-I) Deconvoluted and raw mass spectra for the RRRRY Y200C GFP control.

Supporting Tables

Table S1: Calculated masses of peptides used in this study

Peptide	Mass (Da)
GGGGY	409.4
NNGGY	523.5
NNNNY	637.6
GGGWY	538.6
GGWGY	538.6
RGGGY	508.5
RGRGY	607.7
RRGGY	607.7
RRRGY	706.8
RRRRY	805.9
RRRWY	836.0
RRWRY	836.0
EGGGY	481.5
EGEGY	553.5
EEGGY	553.5
EEEGY	625.6
EEEEY	697.7
EEEWY	754.8
EEWEY	754.8

Table S2: Calculated Potentials of Homology-Based Mutants

Protein	Variant	Electrostatic Energy w/ Lithium (kJ/mol)	Electrostatic Energy w/o Lithium (kJ/mol)	ΔE (kJ/mol)	ΔV (V)	$\Delta\Delta V$ (V)	Conservation Score
abTYR	Full	-309319	-307805	-1515	-15.7	N/A	NA
	Subunit	-59746	-59318	-428	-4.4	N/A	NA
megaTYR	wt	-47096	-47521	426	4.4	N/A	NA
	D16K	-46248	-46743	495	5.1	0.7	1
	D21K	-45541	-46034	493	5.1	0.7	5
	E31K	-46023	-46524	501	5.2	0.8	5
	D55K	-45707	-46414	707	7.3	2.9	3
	D55R	-45928	-46651	723	7.5	3.1	3
	E71K	-45099	-45626	526	5.5	1.0	5
	D79K	-45406	-45921	515	5.3	0.9	5
	E86K	-46072	-46562	490	5.1	0.7	2
	D102K	-46218	-46742	524	5.4	1.0	5
	D123K	-46225	-46758	533	5.5	1.1	1
	D127K	-45648	-46163	515	5.3	0.9	3
	D140K	-45954	-46524	571	5.9	1.5	1
	E141K	-45698	-46310	612	6.3	1.9	2
	E158K	-44989	-45614	626	6.5	2.1	1
	D166K	-45737	-46250	513	5.3	0.9	1
	D167K	-45808	-46349	541	5.6	1.2	5
	D183K	-45796	-46420	624	6.5	2.1	6
	E274K	-45300	-45810	510	5.3	0.9	2
	D275K	-45833	-46348	515	5.3	0.9	4
	E289K	-44505	-45015	509	5.3	0.9	6
	R209H	-47419	-47713	294	3.0	-1.4	6
	R209E	-47882	-48051	170	1.8	-2.7	6
	R280H	-47547	-47935	388	4.0	-0.4	2
	R280E	-48290	-48640	350	3.6	-0.8	2

Table S3: Calculated Potentials of Proximity-Based Mutants

Protein	Variant	Electrostatic Energy w/ Lithium (kJ/mol)	Electrostatic Energy w/o Lithium (kJ/mol)	ΔE (kJ/mol)	ΔV (V)	$\Delta\Delta V$ (V)	Conservation Score
abTYR	Full Subunit	-309319 -59746	-307805 -59318	-1515 -428	-15.7 -4.4	NA NA	NA NA
megaTYR	wt	-47096	-47521	426	4.4	NA	NA
	S54K	-46867	-46340	527	5.5	1.1	1
	D55K	-46219	-45512	707	7.3	2.9	3
	D55R	-46453	-45730	723	7.5	3.1	3
	N57K	-46950	-46540	410	4.2	-0.2	8
	D123K	-46577	-46044	533	5.5	1.1	1
	D140K	-46330	-45759	571	5.9	1.5	1
	E141K	-46124	-45513	611	6.3	1.9	2
	T156K	-47324	-46895	428	4.4	0.0	1
	E158K	-44989	-45614	626	6.5	2.1	1
	D183K	-45796	-46420	624	6.5	2.1	6
	Q187K	-46830	-46343	488	5.1	0.6	1
	R209H	-47419	-47713	294	3.0	-1.4	6
	Q214K	-47162	-46738	424	4.4	0.0	2
	T220K	-46976	-46457	519	5.4	1.0	5

Table S4: Calculated Masses of expressed proteins

Protein	Modification	Unmodified Mass (Da)	Methionine Excision Mass (Da)
Y200C GFP	None	27,549.0	N/A
	RRRRY tag	28,337.0	N/A
	GGSEEEY tag	28,429.9	N/A
paF MS2	None	13,789.0	N/A
megaTYR	Wild Type	35,474.8	35,343.6
	R209H	35,455.8	35,324.6
	D55K	35,487.9	35,356.7
	D55R	35,515.9	35,384.7

Table S5: Calculated masses of Y200C GFP oxidative coupling products

Peptide Coupling Partner	Single Addition Mass (Da)	Double Addition Mass (Da)
None	27,549.0	N/A
GGGGY	27,974.4	28,399.8
NNGGY	28,088.5	28,628.0
NNNNY	28,202.6	28,856.2
GGGWY	28,103.6	28,658.2
GGWGY	28,103.6	28,658.2
RGGGY	28,073.6	28,598.0
RGRGY	28,172.7	28,796.4
RRGGY	28,172.7	28,796.4
RRRGY	28,271.8	28,994.6
RRRRY	28,370.9	29,192.8
RRRWY	28,401.0	29,252.9
RRWRY	28,401.0	29,252.9
EGGGY	28,046.5	28,543.9
EGEGY	28,118.5	28,688.1
EEGGY	28,118.5	28,688.1
EEEGY	28,190.6	28,832.2
EEEEY	28,262.7	28,976.3
EEEWY	28,319.8	29,090.6
EEWEY	28,319.8	29,090.6

Table S6: Calculated masses of paF MS2 oxidative coupling products

Peptide Added	Single Addition Mass (Da)	Double Addition Mass (Da)
None	13,789.00	N/A
GGGGY	14,214.4	14,639.8
NNGGY	14,328.5	14,868.0
NNNNY	14,442.6	15,096.2
GGGWY	14,343.6	14,898.2
GGWGY	14,343.6	14,898.2
RGGGY	14,313.5	14,838.0
RGRGY	14,412.7	14,036.4
RRGGY	14,412.7	14,036.4
RRRGY	14,511.8	15,234.6
RRRRY	14,610.8	15,432.8
RRRWY	14,641.0	15,493.0
RRWRY	14,641.0	15,493.0
EGGGY	14,286.5	14,784.0
EGEGY	14,358.5	14,928.0
EEGGY	14,358.5	14,928.0
EEEGY	14,430.6	15,928.0
EEEEY	14,502.7	15,216.4
EEEWY	14,559.8	15,330.6
EEWEY	14,559.8	15,330.6

Table S7: Y200C GFP Charge Tag Reverse Primers

Y200C GFP Charge Tags	Primer
RRRRY	AGGTCTCCTTTAATAGCGTCGCCG ACGGTGATGGTGATGGTGATGTTTGTACAG
GGSEEEY	AGGTCTCCTTTAATATTCCTCTTCCTCGCTGCCA CCGTGATGGTGATGGTGATGTTTGTACAGTTCA

Table S8: Y200C Charge Tag Forward Primer

primer
AGGTCTCCCATGCGTAAAGGCGAAGAGCTGTTCAGTGG

Table S9: megaTYR Primers

megaTYR Mutants	Forward Primer	Reverse Primer
R209H (1)	GCGTCCACC ACTGGGTAG	GCGATAATGTCG GGCAATCAGGTGCGAC
R209H (2)	GTCGCACCTGATTGC CCGACATTATCGC	CTACCCAG TGGTGGACGC
D55K	AGGTCTCCAAGCGCA ATGCGGCGCACATGTCG	AGGTCTCCGCTTGCT TCCAGGCGGCGTGTGG
D55R	AGGTCTCCCGTCGCAA TGC GGCGCACATGTCG	AGGTCTCCGACGGCT TCCAGGCGGCGTGTGG

Table S10: C6-Amine-DNA

primer
/5AmMC6/AGCAAGGGCGAGGAGCTGTTTCACCG

Chemical Synthesis

Synthesis of Phenol-NHS

500 mg of 3(4-hydroxyphenyl)propanoic acid along with 589 mg (1.7 eq.) *N*-hydroxy-succinamide and 8 mL of THF were added to a flame dried flask that had previously been flushed with nitrogen gas. The flask was then placed on ice, and then 931 mg (1.5 eq.) of DCC (dicyclohexylcarbodiimide) was slowly added to the flask. The flask was then flushed with argon gas, and the reaction was allowed to proceed on ice with gentle stirring for 2 h. The reaction mixture was then filtered through celite and the flow through was diluted in ethyl acetate. Next, the reaction mixture was washed with saturated sodium bicarbonate solution and dried over anhydrous sodium sulfate, after which the solution was evaporated to dryness, resulting in the appearance of a white powder (Phenol-NHS Ester).

Scheme 1: Synthesis of Phenol-NHS Ester

433 *Synthesis of Phenol-DNA* C6-amine DNA from IDT and Phenol NHS Ester were added to a
 434 1:1 mixture of 100mM pH 8.0 aqueous sodium phosphate and DMF. The final concentration
 435 of DNA and phenol-NHS were 100 μ M and 2mM respectively. The reaction mixture was
 436 then placed on ice for 2 h, after which the DNA was then desalted using a NAPTM-25 ST
 437 Cytiva column from Millipore Sigma, and then lyophilized. The resulting powder was then
 438 resuspended in water to a concentration of 1mM and stored at -20°C for later use.

440 Scheme 1: Synthesis of Phenol-DNA

441 Computational methods and electrostatic mapping:

442 analysis was run on the ConSurf web server (<https://consurf.tau.ac.il/>) using the follow-
 443 ing parameters: multiple sequence alignment build using MAAFT, proteins from UNIREF90
 444 database, HAMMR homolog search algorithm, HAMMR E-value: 0.1, number of HAMMR
 445 iterations: 1, Maximal %ID: 95, Minimal %ID: 35, choosing the top 100 sequences, conserva-
 446 tion scores calculated using a Bayesian model, and a Best Fit model of substitution for pro-
 447 teins.^{33–37} Tyrosinase crystal structures from abTYR (PDB ID: 2Y9X) and megaTYR (PDB
 448 ID: 6EI4) were uploaded into the Schrodinger Maestro software suite. A homology model for
 449 cnkTYR was generated using the Zhang lab's I-TASSER server ([https://zhanglab.ccmb.med.u-](https://zhanglab.ccmb.med.u-mich.edu/I-TASSER/)
 450 [mich.edu/I-TASSER/](https://zhanglab.ccmb.med.u-mich.edu/I-TASSER/)) using the default settings and selecting the first output.^{30–32} Following
 451 this, the proteins were then prepared and charges were assigned using the protein preparation
 452 wizard (PPW) tool. First, crystal structures were imported using the default settings under
 453 the import and process tab with the addition of deleting waters more than 3 Å from het
 454 groups. Next, the structures were passed through H-bond Assignment tool under the refine
 455 tab using default settings. Finally, structures were passed through the restrained minimiza-
 456 tion tool with the "Hydrogens Only" box checked, using the OPLS3e force field. abTYR is
 457 a heterooctamer, and megaTYR is a monomer, and so each protein was prepared as such,

and all metal ions other than those found in the type 3 di-nuclear copper active sites were deleted. In addition, a monomeric version of abTYR was constructed by deleting all but one subunit and subsequently put through the same PPW protocol again. Following this, single point mutants of the bacterial tyrosinase were created using the mutation tool found in the Maestro suite and all structures were passed through the second and third steps of the PPW protocol again prior to performing any calculations.

To create the surface ESP plots, the ChimaeraX electrostatic surface tool was used to generate electrostatic surface plots of each of the tyrosinase variants. These structures were then saved as PNG and PDB files. Next, the active site potential was calculated using the following protocol. First, a +1 lithium ion test charge was placed at a distance of 14 Å away from the active site of each enzyme variant. This distance was chosen as it is roughly equivalent to the position of the R-group of the first adjacent residue to the reactive tyrosine in a peptide in the reactive conformation, the conformation in which the tyrosine phenol moiety is placed into the enzyme active site. Then the Macromodel current energy tool was used to calculate the energies of each enzyme variant both with and without the lithium ion test charge. These calculations were performed using the OPLS3e force field with charges coming from the force field, and with water as the solvent. To calculate the ESP, the difference between the electrostatic energy contribution to the total energy for each structure were converted to an electrostatic potential difference using the following formula.

$$V = \frac{(E_{electrostatic_{with-lithium}} - E_{electrostatic_{without-lithium}})(\frac{kJ}{mol}) \times 1000}{96485.3329(\frac{C}{mol})}$$

Tyrosinase-Mediated Oxidative Coupling Reactions of Proteins

Tyrosinase mediated DNA-protein coupling:

DNA (1mM)	GFP (500 μM)	Tyrosinase (200 U/L)	Water
12.5 μL	5 μL	5 μL	27.5 μL

Volumes of each stock solution indicated in the table above were added to an Eppendorf tube with the tyrosinase enzyme added last. The resulting solution was allowed to stand at

room temperature for 1 h minimum before adding 5.0 μ L of a 100 mM aqueous solution of tropolone. The quenched reaction was then diluted with 150 μ L of pH 6.5 50 mM Tris-HCl buffer and analyzed using an SDS-PAGE gel.

Tyrosinase stocks and activity assay:

Agaricus bisporus tyrosinase (abTYR) was obtained from Sigma Aldrich as a lyophilized powder and stored at -20 °C when received. Stock solutions (at 1 mg/mL) were prepared in 50 mM phosphate buffer, in 15% glycerol in water, pH 6.5, stored at -80 °C. *Bacillus megaterium* tyrosinase was obtained by recombinant expression from *E. coli* as described above and stored as a stock solution and -80 °C in a similar buffer after purification. Tyrosinase aliquots were thawed on ice shortly before use and the activity of each new stock was assayed by monitoring conversion of L-Tyrosine to dopachrome via UV-Vis as described previously.²¹ A stability study has indicated that the activity of abTYR aliquots prepared in this manner is stable over months of storage at -80 °C.²¹

Tyrosinase peptide charge screen for subsequent TOF-LC/MS analysis:

Na ₂ HPO ₄ pH 6.5			Protein Nucleophile		
i (mM)	f (mM)	vol (μ L)	i (μ M)	f (μ M)	vol (μ L)
50	10	10.0	500	50.0	5

Peptide (mg/mL)			Tyrosinase			H ₂ O	Total Vol
i (μ M)	f (μ M)	vol (μ L)	i (U/L)	f (U/L)	Vol (μ L)	(μ L)	(μ L)
1000	250	12.5	120	12.0	5.00	17.5	50.0

Volumes of each stock solution indicated in the table above were added to an Eppendorf tube with the tyrosinase enzyme added last. The resulting solution was allowed to stand at room temperature for 30 min before adding 5.0 μ L of a 100 mM aqueous solution of tropolone. The quenched reaction was then diluted with 150 μ L of pH 6.5 50 mM Tris-HCl buffer and submitted to TOF-LC/MS analysis. This standard procedure was followed unless indicated otherwise.

Maleimide Tyrosinase biotin GFP dimer formation

Y200C-EEEEY GFP was capped with maleimide biotin following a previously reported protocol.²³ The resulting solution was then spin-concentrated 6 times using a 50 kDa MWCO spin filter from Milipore to remove excess maleimide biotin. The retained sample was then combined at 10 μ M with 100 μ M of Y200C sfGFP with D55R megaTYR (12 U/L equivalent) for 45 min before quenching and spin concentration using a 50 kDa MWCO spin filter.

Tyrosinase-mediated biotin protein dimer formation

Tyrosinase aliquots were thawed on ice shortly before use and diluted to a 10x stock solution. In the first reaction 10 μ M Y200C GFP-GGSEEEEEY was combined with 100 μ M tyrosine biotin and 200 nM abTYR for 40 min at RT before quenching with tropolone. Solution was then spin-concentrated 6 times using a 50 kDa MWCO spin filter from Milipore to remove excess luciferase. The retained sample was then combined at 10 μ M with 100 μ M of Y200C sfGFP with D55R megaTYR (12 U/L equivalent) for 45 min before quenching and spin concentration using a 50 kDa MWCO spin filter.

Tyrosinase-mediated protein trimer formation

Tyrosinase aliquots were thawed on ice shortly before use and diluted to a 10x stock solution. In the first reaction 10 μ M Y200C GFP-GGSEEEEEY was combined with 100 μ M MYGGS-nanoluciferase and 200 nM abTYR for 40 min at RT before quenching with tropolone. Solution was then spin-concentrated 6 times using a 50 kDa MWCO spin filter from Milipore to remove excess luciferase. The retained sample was then combined at 10 μ M with 100 μ M of Y200C sfGFP with D55R megaTYR (12 U/L equivalent) for 45 min before quenching and spin concentration using a 50 kDa MWCO spin filter.

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Graphical TOC Entry