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Secondary Modification of Oxidatively-Modified Proline N-termini for the Construction of Complex Bioconjugates

*Johnathan C. Maza,[a] Alexandra V. Ramsey,[a] Meire Mehare,[a] Shane W. Krska,[b] Craig A. Parish[b] and Matthew B. Francis [*a,c]*

[a]Department of Chemistry, University of California, Berkeley, California 94720, USA. [b]Discovery Chemistry, Merck & Co., Inc., Kenilworth, New Jersey 07033, USA. [c]Materials Sciences Division, Lawrence Berkeley National Laboratories, Berkeley, California 94720,USA.

*Corresponding author. Email: mbfrancis@berkeley.edu

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Materials and Methods

General Methods and Instrumentation

All reagents were obtained from commercial sources and used without any further purification. EZ-link NHS-biotin and Lucifer Yellow CH, lithium salt, was purchased from Thermo Fisher (Waltham, MA). Aminooxy-5(6)-TAMRA was purchased from Biotium (Fremont, CA). Amino-PEG_4-acid was purchased from BroadPharm (San Diego, CA). Tyrosinase isolated from *Agaricus bisporus* (abTYR, both 25 kU [SKU = T3824-25KU] and 50 kU [SKU = T3824-50KU] were used in these studies) and aldolase isolated from rabbit muscle were purchased from Sigma-Aldrich. Spin concentrators with 10 and 100 kDa molecular weight cutoffs (MWCO) and sterile spin filters with 0.22 μm pores were purchased from Millipore (Billerica, MA). Doubly distilled water (ddH_2O) was obtained from a Millipore purification system.

Liquid chromatography mass spectrometry (LC-MS) analysis. Acetonitrile (Optima grade, 99.9%, Fisher,Waltham, MA), formic acid (1 mL ampules, 99+%, Pierce, Rockford, IL), and water purified to a resistivity of 18.2 MΩ·cm (at 25 °C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA) were used to prepare mobile phase solvents for LC-MS. Electrospray ionization mass spectrometry (ESI-MS) of protein bioconjugates was performed using an Agilent 1260 series liquid chromatograph outfitted with an Agilent 6224 time-of-flight (TOF) LC-MS system (Santa Clara, CA). The LC was equipped with a Proswift RP-4H (monolithic phenyl, 1.0 mm × 50 mm, Dionex) analytical column. Solvent A was 99.9% water/0.1% formic acid and solvent B was 99.9% acetonitrile/0.1% formic acid (v/v). For each sample, approximately 15 to 30 picomoles of analyte were injected onto the column. Following sample injection, a 5-100% B elution gradient was run at a flow rate of 0.30 mL/min over 8 min. Data was collected and analyzed by deconvolution of the entire elution profile in order to provide reconstructed mass spectra that are representative of the entire sample using Agilent MassHunter Qualitative Analysis B.05.00. Percent modification was determined through integration of MS peaks using opensource Chartograph software (www.chartograph.com). The integration of the completely unmodified protein peak served as an internal standard in determining the percent modification.

UV-VIS measurements. A NanoDrop 1000 (Thermo) was used to quantify the samples based on absorbance values at 280 nm (or 488 nm for sfGFP).

Fluorescence microscopy. Fluorescence microscopy was performed on an EVOS M7000 imaging system (ThermoFisher Scientific) using the phase and GFP channels.

Experimental Procedures

abTYR-mediated oxidative coupling of p-cresol and biotin-phenol to proline N-termini. To a 10 μM protein solution in 20 mM phosphate buffer (pH = 6.5) was added the phenol coupling partner to a final concentration of 100 μM. Resin-bound abTYR (prepared with a 2 mg/mL solution of abTYR) was added at a 1:10 dilution. Thus, for a 500 μL coupling reaction, 50 μL of abTYR resin (2 mg/mL) was added to 450 μL of coupling mix. The reaction was allowed to proceed at room temperature for 30 min. Reactions were stopped by spinning the full reaction volume through a 0.22 μm centrifugal filter (to remove the abTYR resin), adding TCEP (final concentration of 1.9 mM), and allowing reactions to stand at RT for 1 min. Excess biotin-phenol was removed via spin concentration against 10 kDa MWCO filters with 20 mM phosphate buffer at pH 7.2. The resulting samples were analyzed using ESI-TOF MS.

Oxime or hydrazone ligation onto o-quinone-proline N-termini. To a 10 μM solution of *o*-quinone-modified proline N-terminal protein in 50 mM phosphate buffer (pH = 5.0) was added the corresponding alkoxyamine or hydrazine coupling partner to a concentration of 5 mM. The reaction was allowed to proceed at RT for overnight (~20-22 h). Excess small molecule was purified away via spin concentration against 10 kDa MWCO filters with 10 mM bisTris buffer at pH 7.2. The resulting samples were analyzed using ESI-TOF MS.

Disulfide exchange with Ellman's reagent. Free cysteines on pro-TMV and aldolase were protected from potential modification by disulfide formation with Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB). To a solution of each protein (50 μL of a 100 μM solution) was added DTNB (11 μL of a 20 mM solution in 100 mM phosphate buffer, pH 7.2 with 1 mM EDTA). The reaction mixture was incubated at rt for 15 min, upon which the solution turned from clear to bright yellow, indicating the protection of reactive cysteine residues. Excess DTNB was removed by repeated (3-6 times) centrifugal filtration with a 10 or 100 kDa MWCO membrane against 20 mM phosphate buffer at pH 7.0. After reaction, the Ellman's reagent protecting group was removed via addition of TCEP (final concentration of 1.9 mM) and incubation at RT for 5 min.

BCA assay for determination of protein yield. N-terminal pro-sfGFP was modified with *p*-cresol as described above. After modification and purification using 10 kDa MWCO filters, two portions were modified with CH_3ONH_2 or lucifer yellow hydrazine as described above and purified using 10 kDa MWCO filters. As controls, unmodified pro-sfGFP was incubated in the same reaction buffers for the same amount of time at each step. To assess protein yields, a BCA assay (Thermo) was performed on the resulting products. These results are summarized in SI Fig S1.

Stability study on oxime or hydrazone modified o-*quinone-pro-sfGFP. Ortho*-quinone-pro-sfGFP was prepared as described above. Synthesis of large-scale oxime or hydrazone-modified o-quinone-pro-sfGFP was performed as described above, using 10 μM of o-quinone-pro-sfGFP and 5 mM of either CH3ON2 or lucifer yellow all in 50 mM pH 5.0 phosphate

buffer at a scale of 250 µL. The reactions were allowed to sit at room temperature for 24 h and then diluted with 20 mM pH 7.2 phosphate buffer and spin concentrated a total of 5 times, until no more excess lucifer yellow could be seen in the flow through. The purified reactions were brought to 50 µL volumes (~50 µM protein product) and a portion was analyzed using ESI-TOF MS to determine percent abundance of pro-sfGFP, o-quinone-pro-sfGFP, and oxime or hydrazone product using the open-access chartograph website (initial arrow in SI Fig S3). Following purification, 10 µM portions of the reaction products were prepared in 20 mM phosphate buffer at pH 7.2 and were incubated for 24 h at 4 ºC, room temperature, and 37 ºC. An additional portion was prepared the same way but with 10 mM of reduced glutathione added to it and allowed to sit for 24 h at room temperature. Following the 24 h incubations, all stability tests were filtered through a 0.22 µm cellulose acetate centrifugal filter and were then analyzed using ESI-TOF MS as described above.

Preparation of tyrosinase resin (adapted from GE Healthcare protein coupling protocol). NHS-Activated Sepharose 4 Fast Flow resin (GE Healthcare) was washed nine times with 1 mM HCl, followed by two washes with 50 mM phosphate buffer at pH 8. The resin was re-suspended in 2 mg/mL tyrosinase (from Agaricus bisporus, Sigma Aldrich, CAS 9002-10-2) in 50 mM phosphate buffer at pH 8. The resin was rotated end-over-end overnight at 4°C and then drained and washed two times with 50 mM phosphate buffer at pH 8. Unreacted NHS was blocked for 2 h at RT with ethanolamine (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3). The ethanolamine solution was drained, and the resin was washed 3 times with 0.1 M Tris-HCl buffer at pH 8.5 and 3 times with 0.1 M acetate buffer, 0.5 M NaCl at pH 4.5. This was repeated three times for a total of four wash cycles. The resin was re-suspended in 20 mM PB at pH 6.5 and stored at 4°C until use.

Reaction rate test for immobilized abTYR resin. Para-cresol (1.5 mM) was reacted with l-proline methyl ester (150 mM) at pH 6.5 (20 mM phosphate buffer) in the presence of tyrosinase resin (10% v/v). To determine the concentration of product formed, A_{520} was obtained at time points between 1 and 17 minutes. Tyrosinase resin was removed from solution via centrifugation, and the reaction was diluted 10-fold prior to absorption measurements. The extinction coefficient for the product (ε_{520} = 3500 M-1cm-1)[1] was used to convert A_{520} to concentration, and then the reaction rate was determined by plotting product concentration versus time (see SI Fig S4).

Reusability studies for abTYR resin. After carrying out a reaction rate test (described above), the tyrosinase resin was collected and washed four times with 50 mM phosphate buffer at pH 8. The washed resin was resuspended in phosphate buffer and then used to catalyze the next reaction rate test. This was repeated until the resin had been used for six reaction rate tests (see SI Fig S6).

Stability studies for abTYR resin. The reaction rate test (described above) was carried out multiple times over the course of 25 days. Fresh resin was used during each test, but all resin originated from a single batch prepared on “day 1” and stored at 4°C over the course of the study (see SI Fig S6).

Aldolase activity assay. The proline N-terminus of aldolase was modified using the oxidative coupling procedure described above. A portion of the *o*-quinone-modified aldolase was then modified with methylalkoxyamine. After purification, all aldolases were stored in 20 mM phosphate buffer at pH 7.2. To assess activity, *o*-quinone-modified aldolase, oxime-modified aldolase, and unmodified aldolase were diluted to 20 nM in assay buffer (20 mM phosphate buffer at pH 8.5 with 3 µM DTT) in triplicate in a clear bottom 96 well plate. To this solution was added NAD^+ to a final concentration of 1 mM and GAPDH to a final concentration of 1.35 µM. The plate was pre-warmed to 37 ºC, and then fructose bisphosphate (FBP) was added to the wells to a final concentration of 100 µM, bringing the total volume to 200 µL. Blank wells were set up containing all reagents except aldolase, as well as assay buffer only wells. Immediatley after adding FBP to the wells, the absorbance at 340 nm was monitored every 2 min for 5 h at 37 ºC in a Tecan microplate reader.

Modification of alkoxyamine resin with o*-quinone-pro-sfGFP.* To begin, ~25 µL of settled alkoxyamine-PEGA resin (stored as a slurry in DMF) was placed into a clean 0.6 mL eppendorf tube. Using gel loading pipette tips, the DMF was removed, avoiding the settled beads. The resin was then washed three times with 200 µL portions of 100 mM phosphate buffer at pH 5.0, spinning down the beads at 14.1 rcf for 1 min before removing the rinse. To the beads was added 40 µL of 50 µM *o*-quinone-pro-sfGFP in 50 mM phosphate buffer at pH 5.0. As controls, beads were brought up in either 40 µL of 50 mM phosphate buffer at pH 5.0 or 40 µL of 50 µM pro-sfGFP in 50 mM phosphate buffer at pH 5.0. As a final control, 25 µL of the amino PEGA resin, from which the alkoxyamine PEGA resin was prepared, was prepared similarly to the alkoxyamine resin. To this amino PEGA resin was added 40 µL of 50 µM *o*-quinone-pro-sfGFP in 50 mM phosphate buffer at pH 5.0. The beads were covered in tin foil and allowed to rotate for 24 h in an end-over-end rotator. The next day, the reaction supernatant was removed, and the beads were washed three times with 400 µL of 20 mM phosphate buffer at pH 7.0, similar to the wash steps described above. The beads were resuspended in 400 µL of 20 mM phosphate buffer at pH 7.0 and imaged using the phase and GFP settings on an EVOS fluorescent microscope (ThermoFisher).

Expression of N-terminal pro-sfGFP. Expression was carried out as previously reported.[2] Purified protein was spin concentrated into 20 mM phosphate buffer at pH 7 using 10DG Desalting Columns (Bio-Rad) prior to reaction.

Expression of N-terminal pro-TMV. Expression and purification was carried out as previously reported.[3] Purified protein was spin concentrated into 20 mM phosphate buffer at pH 7 using 10k MWCO spin filters prior to reaction.

Generation of sfGFP-Y182C. Around-the-horn mutagenesis was performed on the DNA sequence for met-sfGFP using the primers below, which coded for mutagenesis of tyr 182 to a cys residue. Expression was carried out similarly to the

pro-sfGFP.

Forward Primer: GCTGGCTGATCACTGCCAGCAAAACACTCC

Reverse Primer: GGAGTGTTTTGCTGGCAGTGATCAGCCAGC

Procedure for expression and purification of pCBD-TEV-TrxA protein. A derivative of our previously reported N-terminal proline Chitin-Binding Domain(CBD)-TEV-TrxA fusion protein was assembled using Gibson cloning.[4] The following gene block was purchased from International DNA Technologies, Inc. (IDT), where the pro-CBD domain is in blue, the TEV cleavage site is in pink, and the TrxA solubilizing domain is in green.

tgagcggataacaattcccctctagaaataattttgtttaactttaagaaggagatataccATGCCAACGACTC–
CAGTCCCAGTAAGCGGCTCTCTGGAAGTCAAAGTCAACGATTGGGGGTCTGGGGCCGAATATGACGTGACATTA–
AATTTAGACGGTCAGTACGACTGGACAGTAAAGGTGAAACTTGCTCCGGGGGCAACCGTGGGCAGCTTCTGGAGC–
GCAAATAAACAGGAAGGCAATGGGTATGTTATCTTTACTCCGGTATCGTGGAACAAAGGACCAACCGCGACTTTC–
GGTTTCATCGTCAACGGACCACAGGGTGACAAAGTCGAGGAGATCACCTTAGAGATTAACGGCCAGGTTATTGGAG–
GCTCAGGAGGCTCGgaaaacctgtattttcagggtGGAGGCTCAGGAGGCTCGATGTCGGATAAAATTATCCATTT–
AACGGATGATAGTTTTGATACTGACGTGTTGAAGGCCGACGGGGCCATCTTGGTAGACTTCTGGGCAGAGTGGTGCG–
GTCCGTGTAAGATGATTGCACCCATTTTAGATGAAATTGCTGATGAGTACCAAGGGAAGTTGACAGTTGCTAAATT–
GAATATCGACCAGAATCCAGGGACCGCACCCAAATACGGCATTCGTGGAATCCCTACATTGTTACTTTTCAAGAATG–
GGGAGGTCGCTGCCACTAAGGTTGGCGCTCTTAGCAAGGGGCAATTAAAAGAATTTTTGGACGCAAACTTGGCGctc–
gagcaccaccaccaccaccactgag

When inserted into a pET 28b cloning vector precut with XbaI and XhoI restriciton enzymes using the Gibson assembly method, the gene block resulted in an in-frame coding sequence for the following protein:

(M)PTTPVPVSGSLEVKVNDWGSGAEYDVTLNLDGQYDWTVKVKLAPGATVGSFWSANKQEGNGYVIFT–
PVSWNKGPTATFGFIVNGPQGDKVEEITLEINGQVIGGSGGSENLYFQGGGSGGSMSDKIIHLTDDSFDTD–
VLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKV–
GALSKGQLKEFLDANLALEHHHHHH

Where the initial met residue is clipped. Following sequence confirmation and transformation into BL21(DE3) Star *E. coli*, a 10 mL overnight culture was grown in Luria-Burtani (LB) media containing 1 mM kanamycin at 37 °C. The full 10 mL culture was then added to 1 L of 2XYT media and grown at 37 °C to an OD600 of 0.6 - 0.8. Expression was induced with a final concentration of 1 mM IPTG. After 20 h, the cells were collected by centrifugation at 8000*x*g for 15 min at 4 °C. After freezing at -80 ºC, cell pellets were resuspended in 20 mL of an equilibration buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole at pH = 7.4) and then lysed via sonication for 20 min at 70% amplitude. Cells were loaded onto 3 mL of pre-equilibrated Ni-NTA spin columns (Thermo) and allowed to mix in an end-over-end rotator for 30 min at 4 ºC. After mixing, the bound protein was washed with 10 portions of two resin bed volumes (6 mL) of wash buffer (20 mM sodium phosphate, 300 mM NaCl, 25 mM imidazole at pH = 7.4) and subsequently eluted with three portions of one resin bed volume (3 mL) of elution buffer (20 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole at pH = 7.4). The purified pro-CBD-TEV-TrxA was then buffer exchanged into 20 mM phosphate buffer at pH 7.2 prior to reaction.

Synthetic Procedures

Synthesis of biotin-phenol. Biotin-phenol was prepared as previously reported.[4] To a 40 mM solution of tyramine (1 eq, Sigma Aldrich) in dry DMF was added E-Z link biotin-NHS (1.1 eq, Thermo) to a final concentration of 44 mM. The reaction was vortexed overnight and analyzed by LC-MS to confirm amide bond formation. To hydrolyze any remaining NHS ester, portions of this reaction solution were diluted with water to a final concentration of 2 mM and allowed to stand for 48 h before use in subsequent labeling reactions. ESI-Q-TRAP MS: *m/z* calc'd for $C_{18}H_{26}N_3O_3S$ $[M+H]^+$ 364.16, observed 364.7.

Synthesis of alkoxyamine-PEG$_4$-aniline. To a flame dried 25 mL round bottom and stir bar was added 144 mg of (Boc-aminooxy)acetic acid (0.754 mmol, 1 equiv), which was then dissolved in 10 mL of dry DCM. To this solution was added 176 mg of N,N'-dicyclohexylcarbodiimide (0.852 mmol, 1.13 equiv) and 87 mg of N-hydroxysuccinimide (0.754 mmol, 1 equiv). This mixture was allowed to stir for 15 mins at room temperature. After the 15 mins, the reaction was filtered through a 0.22 µm PVDF syringe filter into a flame dried 20 mL scin vial. To this vial was added 200 mg of amino-PEG$_4$-acid (0.754 mmol, 1 equiv, BroadPharm, San Diego) and 280 µL of triethylamine (2.013 mmol, 2.67 equiv). The reaction was allowed to stir for 2 h at room temperature. After 2 h, the reaction was rotovapped and dried under vacuum. This crude reaction (0.754 mmol, 1 equiv) was then dissolved in 10 mL of dry DCM. To this mixture was added 176 mg of N,N'-dicyclohexylcarbodiimide (0.852 mmol, 1.13 equiv) and 87 mg of N-hydroxysuccinimide (0.754 mmol, 1 equiv) and the reaction was allowed to stir for 15 min at room temperature. After the 15 min, the reaction grew cloudy, and it was then filtered through a celite plug into a flame dried 50 mL round bottom. To the filtrate was added 119 µL of 4-(2-aminoethyl)aniline (0.905 mmol, 1.2 equiv) and 280 µL of triethylamine (2.013 mmol, 2.67 equiv). The reaction proceeded overnight and was then diluted with 10 mL of

DCM prior to filtering through a celite plug. The reaction was rotovapped and purified using silica gel chromatography in 8% MeOH in DCM. Fractions were collected and rotovapped, yielding 146 mg (0.262 mmol, 35% yield) of a yellow oil. 1H NMR was used to confirm the product (400 MHz, MeOD): δ 6.95 (d, 2H, J = 8.36), 6.68 (d, 2H, J = 8.40), 4.25 (s, 2H), 3.68 (t, 2H, J = 6.12), 3.42 (t, 2H, J = 5.44), 3.34 (s, 16H), 2.65 (t, 2H, J = 7.52), 2.40 (t, 2H, J = 6.12), 1.47 (s, 9 H). ESI-Q-TRAP MS: m/z calc'd for $C_{26}H_{44}N_4O_9$ ([MH]+) 557.31, found 557.3 m/z. To Boc-deprotect the compound, 98 mgs of the above product was dissolved in 3 mL of a 95% TFA, 2.5% H_2O, and 2.5% TiPS solution. This deprotection was allowed to stir at room temperature for 30 mins, and then the TFA was blown off using N_2 followed by rotovapping. The deprotected product was dissolved in water, and excess acid was neutralized using 5 M NaOH until a pH of 7 was obtained. This was then used in subsequent oxime ligation reactions.

Synthesis of alkoxyamine-PEGA resin. To begin, (Boc-aminooxy)acetic acid was activated with NHS. To a 20 mL scin vial was added 191 mg of (Boc-aminooxy)acetic acid (1.0 mmol, 1 equiv), 233 mg of N,N'-dicyclohexylcarbodiimide (1.13 mmol, 1.13 equiv), and 115 mg of N-hydroxysuccinimide (1.0 mmol, 1 equiv). These were then suspended in 10 mL of DCM and allowed to stir for 15 mins at room temperature, after which the reaction mixture was filtered through a 0.22 µm PVDF syringe filter and rotovapped to dryness. To a 2 mL Poly-Prep chromatography column (Bio-Rad) was transferred a slurry of amino PEGA resin (Novabiochem) in DMF until ~1.5 mL of settled resin was measured (~0.1 g, 0.04 mmol total loading). The resin was rinsed three times with 2 mL of DMF. The NHS-activated (Boc-aminooxy)acetic acid was then dissolved in 1 mL of DMF and added to the amino PEGA resin. To this mixture was added 1 mL of TEA, and the reaction was allowed to rotate end-over-end overnight at room temperature. The next day, the resin was washed with alternating rinses of 2 mL of DMF and 2 mL of water, three times, ending with a DMF wash. The Boc-protected aminooxy-PEGA resin was resuspended in fresh 2 mL of DMF. To this was added 2 mL of TFA, dropwise, to Boc deprotect the resin. The deprotection reaction was allowed to rotate end-over-end at room temperature for 1 h, after which the resin was drained and rinsed three times with 2 mL of DCM, followed by three rinses with 2 mL of water. After these rinses, any remaining acid was neutralized via the addition of 2 mL of 1 M NaOH brine. The beads were then brought to pH ~ 5.0 by rinsing them five times with 2 mL portion of 100 mM phosphate buffer at pH 5.0. The pH of the beads was verified by suspending a small portion of the beads in water and blotting the water on pH paper. Once the desired pH was obtained, the beads were drained of any aqueous solution and stored for long-term in DMF at 4 ºC. Successful modification of the beads with the alkoxyamine was verified via reaction with *o*-quinone-pro-sfGFP as detailed in the manuscript.

Supporting Information References

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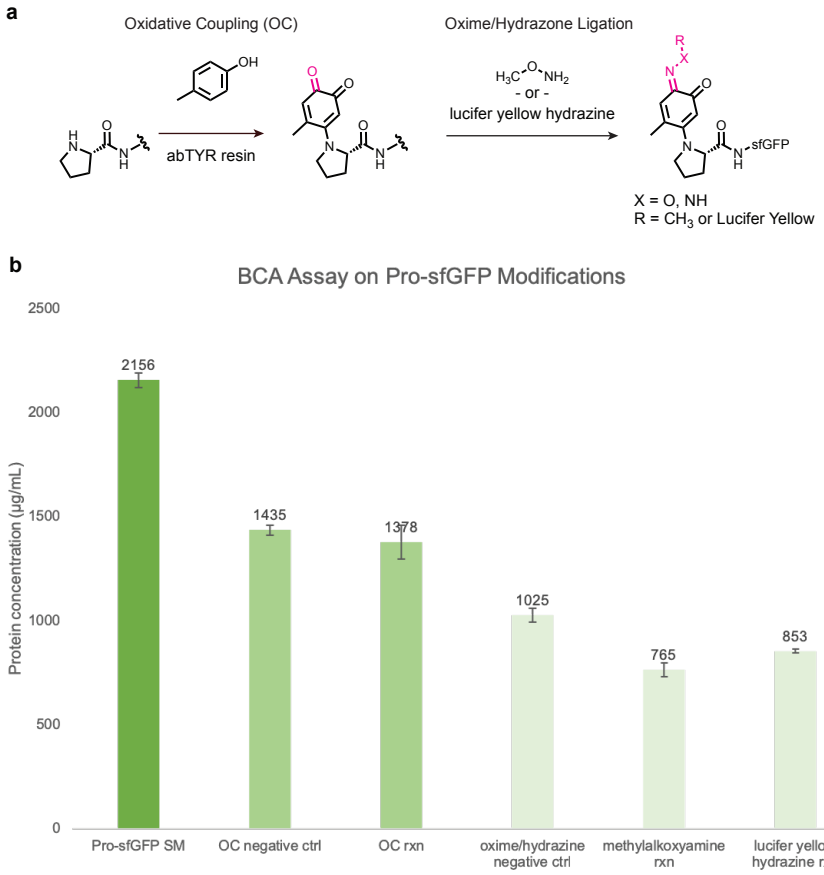
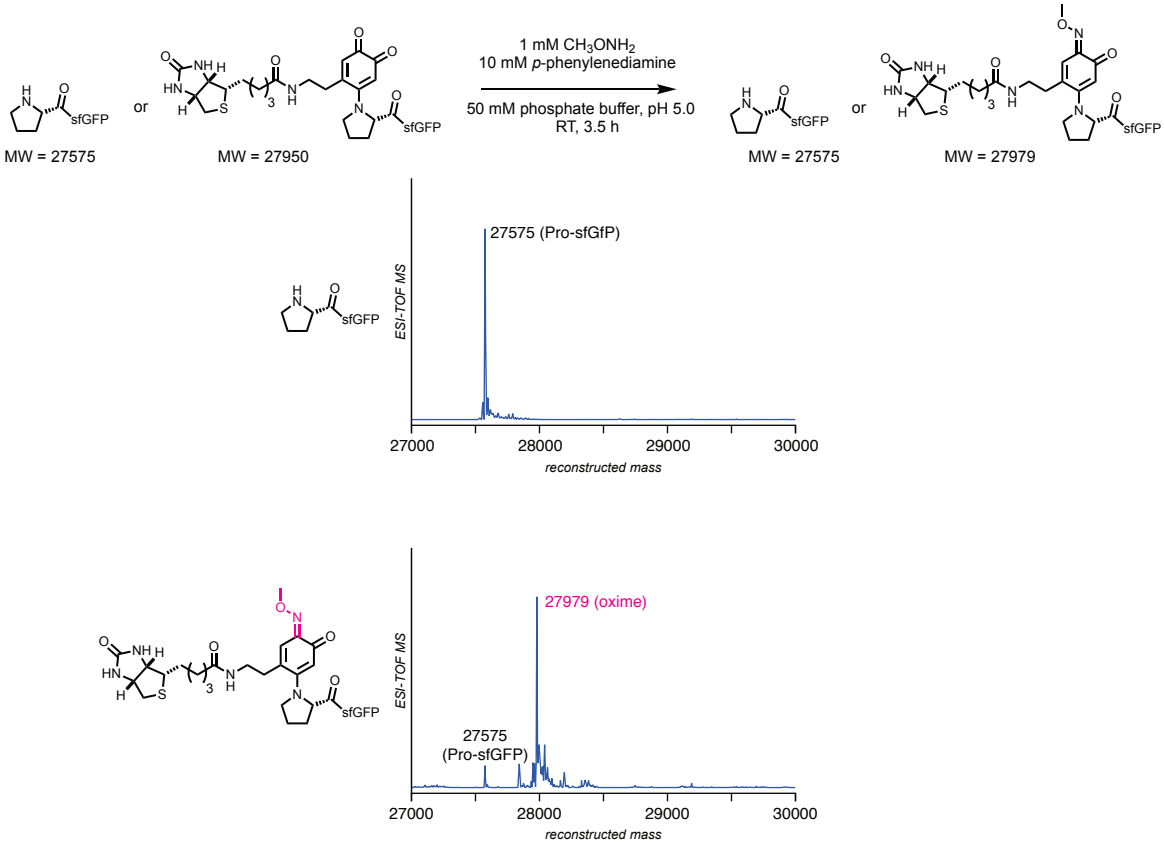
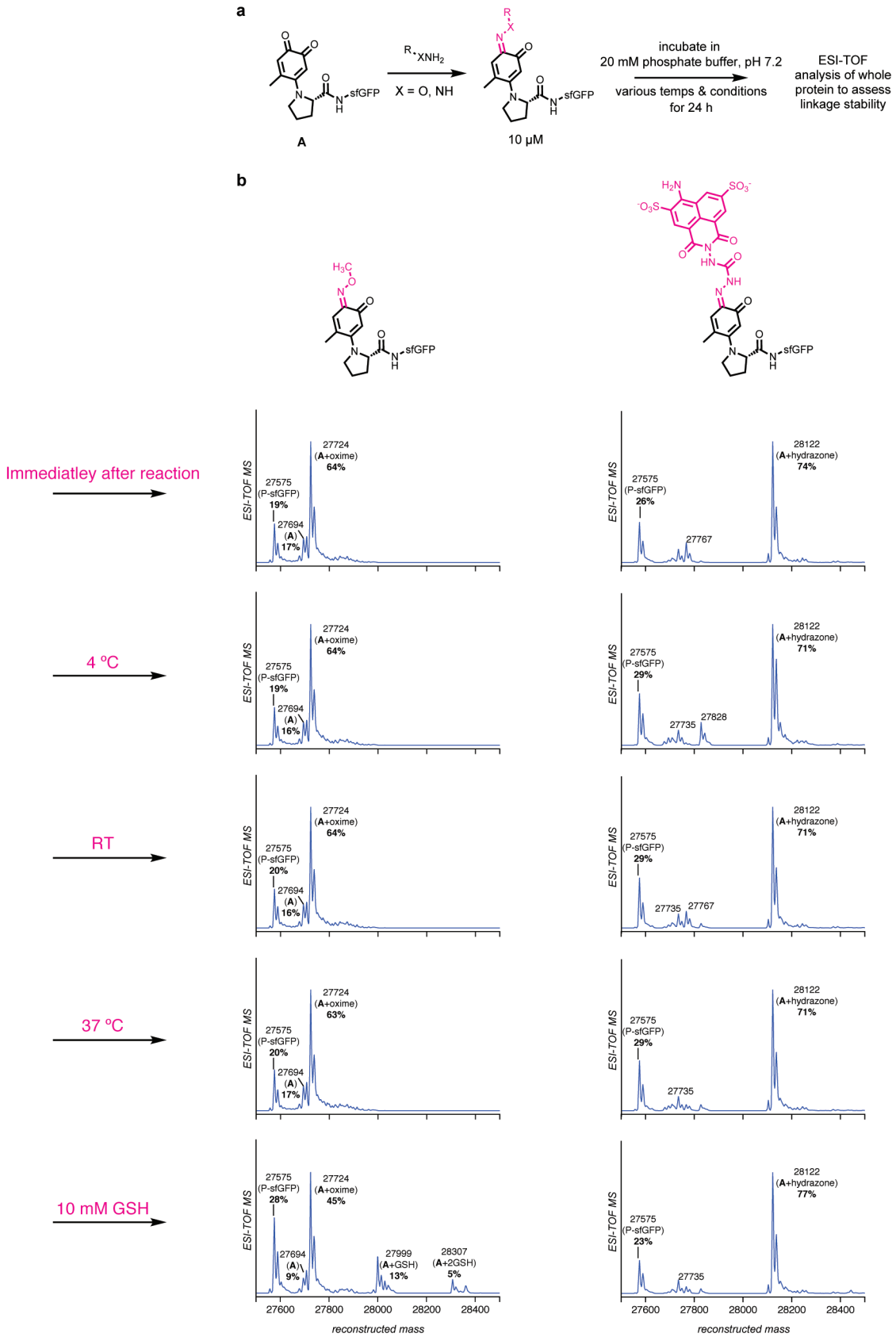


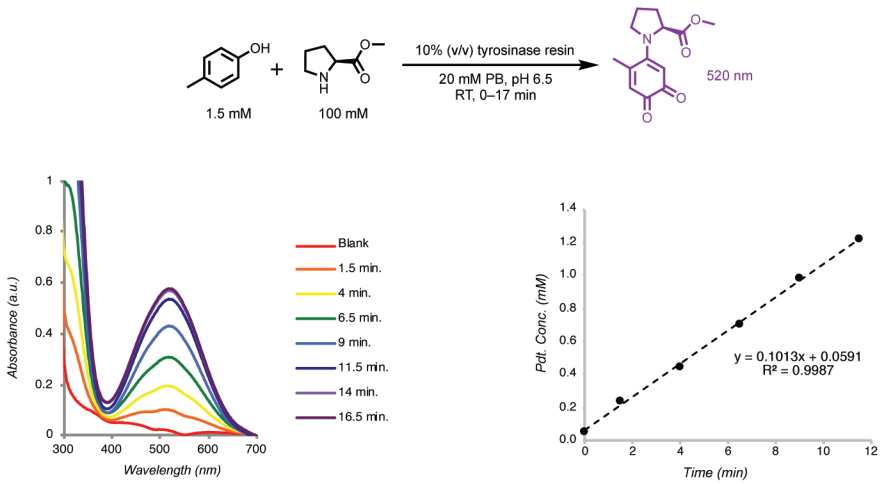
Supporting Information Figure S1. BCA assay assessing protein yields over the course of the two-step reaction procedure. (a) General scheme for the coupling procedure. First, an N-terminal oxidative coupling reaction was performed using the resin-bound abTYR and p-cresol, as described above. Next, the o-quinone-modified pro-sfGFP underwent oxime or hydrazone formation with methylalkoxyamine or lucifer yellow hydrazine, as described above. After each step, the protein product was purified by spin concentrating in 10k MWCO spin filters against 20 mM phosphate buffer at pH 7.2. Negative controls, which were pro-sfGFP incubated in the same buffer conditions for the same length of time as the reactions, were carried through the same workup procedures. (b) A BCA assay was performed to quantify the concentration of protein after each step. Losses in protein quantity are observed at each step, however, they are independent of the modification event, as evidenced by a similar drop in protein quantity for the pro-sfGFP negative controls. A small amount of protein loss is observed in the case of the final step, as evidenced by the slight difference in protein quantity between the oxime/hydrazine buffer negative control and the reactions.



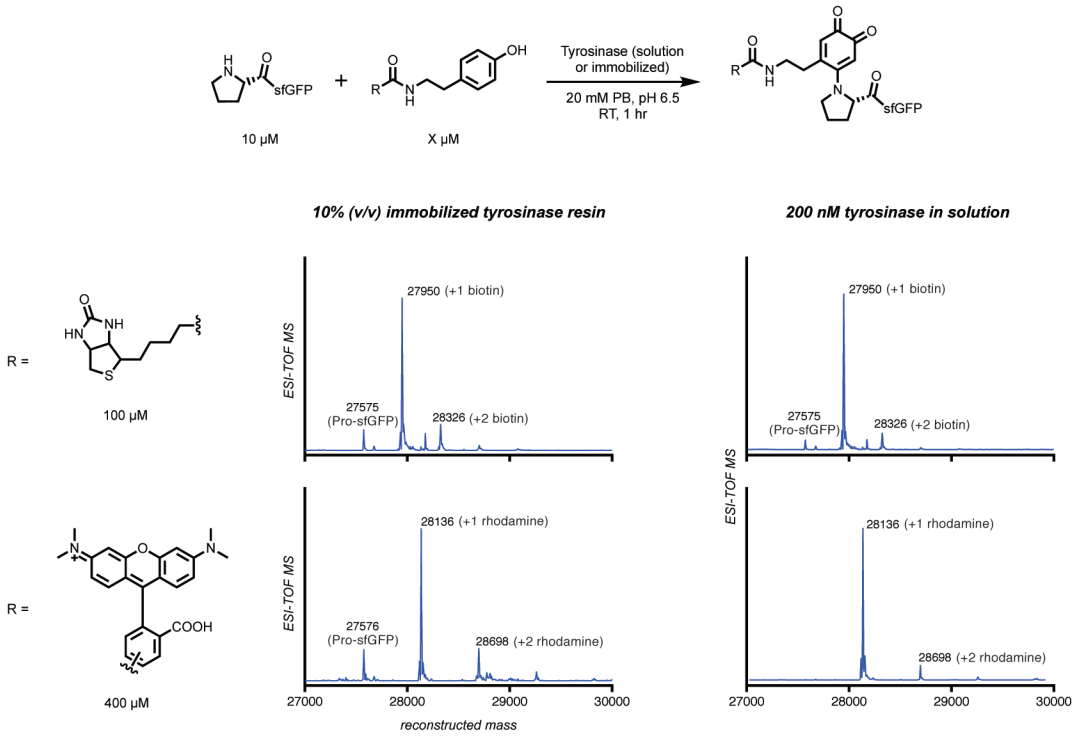
Supporting Information Figure S2. Acceleration of oxime ligation reactions using covalent catalysis. Test couplings were performed on 10 μM of pro-sfGFP or *o*-quinone-biotin-modified pro-sfGFP in the presence of 1 mM CH_3ONH_2 and 10 mM p-phenylenediamine. Reactions were run for 3.5 h in 50 mM phosphate buffer at pH 5.0. Oxime ligation was complete in 3.5 h only when the o-quinone moiety had previously been installed on pro-sfGFP.



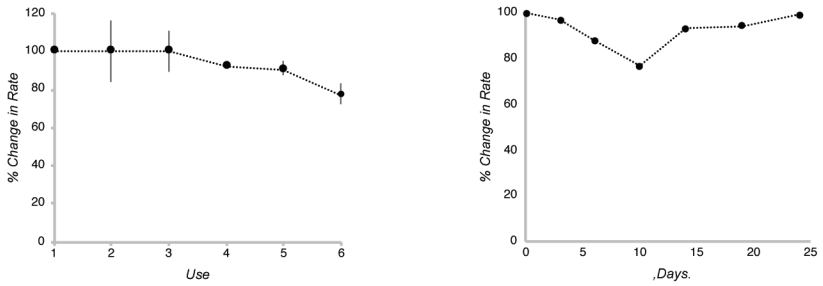
Supporting Information Figure S3. (a) To asses the stability of the oxime and hydrazone linkages under various conditions, a large batch of *o*-quinone-pro-sfGFP modified with CH_3ONH_2 or lucifer yellow hydrazine was prepared. These were then incubated for 24 h in 20 mM phosphate buffer at pH 7.2 at various conditions. (b) Both oxime and hydrazone linkages were stable for 24 h in temperatures ranging from 4 - 37 ºC. The addition of 10 mM reduced glutathione (GSH), a biologically relevant thiol nucleophile, had no effect on the hydrazone linkage. The oxime linkage showed a small amount of reversion to the unmodified *o*-quinone adduct in the presence of this nucleophile (~19%), which then underwent secondary additions with GSH. No additions of GSH to the oxime modified *o*-quinone ring were observed.



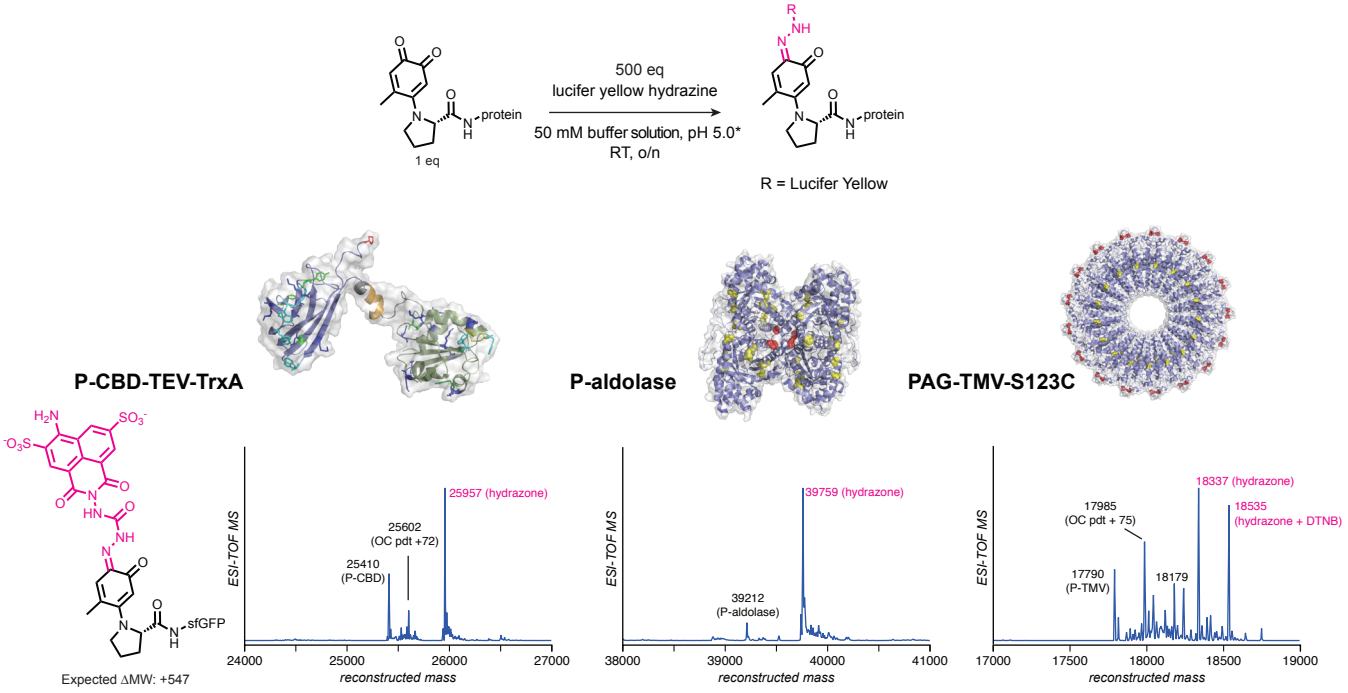
Supporting Information Figure S4. Determination of small molecule oxidative coupling reaction rate in the presence of immobilized tyrosinase. Couplings were performed with 1.5 mM *p*-cresol and 100 mM l-proline methyl ester in the presence of 10% (v/v) immobilized tyrosinase resin. Product concentration was determined by obtaining the UV-vis absorbance of the reaction at time intervals between 0 and 17 minutes. Absorbance at 520 nm was converted to concentration using the extinction coefficient for the product (ε_{520}= 3,500 $M^{-1}cm^{-1}$).



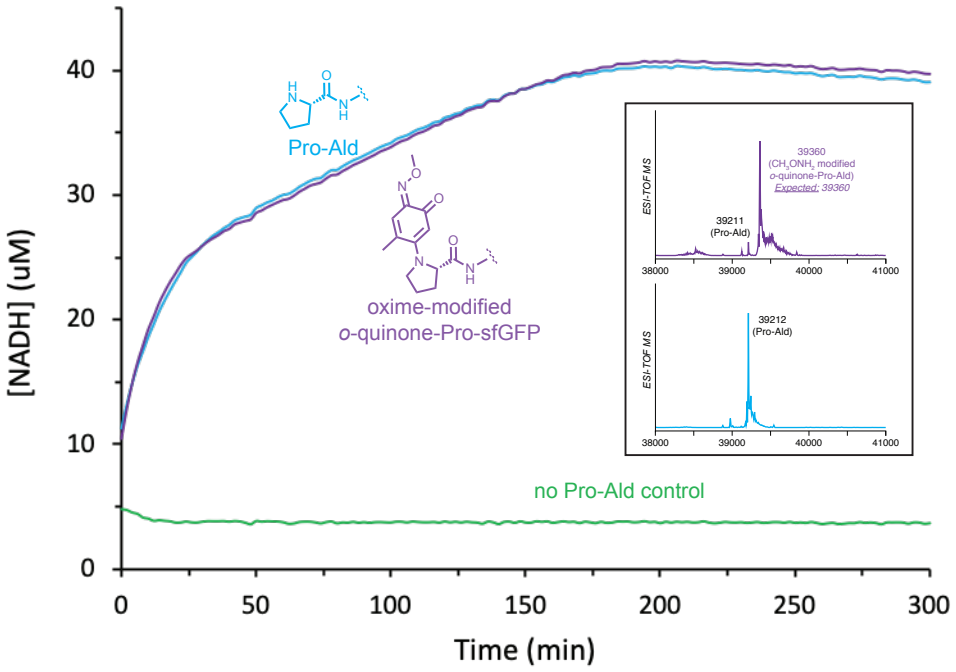
Supporting Information Figure S5. Modification of pro-sfGFP with both immoblized tyrosinase resin and tyrosinase in solution. Pro-sfGFP at 10 uM was reacted with either 100 µM biotin-phenol or 400 µM rhodamine-phenol in the presence of 10% (v/v) immobilized tyrosinase resin or 200 nM solution tyrosinase. Reactions were run for 1 h in 20 mM phosphate buffer at pH 6.5. For samples catalyzed by immobilized tyrosinase resin, reactions were quenched by removing the resin with a 0.22 µm cellulose acetate spin filter. For samples catalyzed by solution tyrosinase, reactions were quenched by the addition of tropolone and TCEP (final concentrations of 1.9 mM for each).



Supporting Information Figure S6. Reusability (left) and long-term storage (right) of immobilized tyrosinase resin. Rates for each point were determined using the method previously described in Fig. S4, and percent change in rate was found by comparing to the rate for the first use (for reusability studies) or to the the rate on the first day (for storage studies). For reusability studies, the tyrosinase resin was collected after each reaction, washed four times with 50 mM phosphate buffer at pH 8, and then used to catalyze the next reaction. Error bars represent standard deviation for n = 2. For storage studies, fresh resin was used during each test, but all resin was from a single batch prepared on "day 1" and stored at 4°C over the course of the study.



Supporting Information Figure S7. Modification of various *o*-quinone-pro N-termini. To 1 eq (i.e. 10 μM) of *o*-quinone-proline N-terminal protein was added 500 eq (i.e. 5 mM) of lucifer yellow hydrazine. The reaction mix was allowed to react at room temperature in 50 mM phosphate buffer at pH 5.0 (*pH = 5.44 for *o*-quinone-pro-CBD). Analysis via ESI-TOF mass spectrometry was used to confirm successful hydrazone ligation. Conversion to the expected product was observed with pro-CBD and aldolase that had been previously modified with *o*-quinone. While *o*-quinone-pro-TMV also underwent conversion, a high degree of protein decomposition was observed in the ESI-TOF trace, as indicated by the large growth in the baseline noise peaks.



Supporting Information Figure S8. Aldolase activity assay on oxime-modified *o*-quinone-pro-sfGFP. Briefly, 20 nM of either unmodified pro-aldolase or o-quinone-pro-aldolase modified with methylalkoxyamine, was incubated with 1 mM of NAD^+, 1.35 µM ofGAPDH, and 100 µM of fructose bisphosphate all in 20 mM potassium phosphate buffer at pH 8.5 with 3 µM DTT. The reaction was monitored at 37 ºC, and absorbance readings at 340 nm were taken every 2 mins. No difference in the rate of production of reduced NADH was observed between the two aldolase samples tested, indicating that the sequential oxidative coupling and oxime ligations did not negatively perturb aldolase function. Inset shows ESI-TOF traces for modified and unmodified aldolase samples used in this activity assay.