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Supporting information

A click chemistry approach to site-specific immobilization of a small laccase enables efficient direct electron transfer in a biocathode

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Materials and Methods

Chemicals and Bacterial Strains

Ultrapure O_2 and N_2 (>99.99%) were purchased from Acetylene Oxygen Co. (Harlingen, TX). Indium-tin oxide (ITO) electrodes (surface resistance 5 – 15 Ω), buckypaper and silver conductive epoxy glue were purchased from Delta Technologies, Ltd. (Loveland, CO), NanoTechLabs, Inc. (Yadkinville, NC) and MG Chemicals (Surrey, Canada), respectively. The unnatural amino acid 4-azido-L-phenylalanine was purchased from WuXi AppTec.

All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO), VWR International (Radnor, PA), or Thermo Fisher Scientific (Waltham, MA) unless otherwise specified. *Escherichia coli* strains DH5α (Invitrogen, Carlsbad, CA) and BL21(DE3) (New England Biolabs, Ipswich, MA) were used for recombinant DNA cloning and recombinant protein expression, respectively.

Plasmid Construction

The sequences of primers used in this study are shown in Table S1. To construct SLAC TAG mutants, the SLAC gene from pSlac1 (a gift from Prof. Gerard Canters, Leiden University, Netherlands)[1], was modified by site-directed mutagenesis (overlap extension PCR) using the primers Slac-F/SlacXXXTAG-R and SlacXXXTAG-F/Slac-R, and the mutant gene was inserted into SH3-Slac[2] between the NheI and XhoI sites to replace Slac WT with an amber stop codon TAG at the selected sites. All constructs contain a small SH3 domain at the N-terminus and a 6xHis tag at the C terminus.

Protein Expression and Purification

E. coli BL21(DE3) was co-transformed with each SH3-SLAC construct (TAG mutants or WT) and pEVOL-AzFRS[3] and plated on a Luria−Bertani (LB) agar plate containing 50 μg/ml kanamycin (Kan) and 34 μg/ml chloramphenicol (Cm). The next day, a single colony was inoculated into 5 mL of LB broth containing 50 μg/ml Kan and 34 μg/ml Cm, and was grown at 37 °C with shaking at 250 rpm until an optical absorbance (OD_{600}) of ~0.6 was obtained. Thereafter, the 5 mL culture was transferred to 400 ml of LB broth containing 50 μg/ml Kan, 34 μg/ml Cm and 0.25 mM $CuCl_2$ (for SLAC enzyme), and the growth was continued until an OD_{600} of ~1.0 was obtained. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM), arabinose (0.02%, w/v) and AzF (1 mM) followed by shaking incubation at 21 °C for 15 h. After expression, cells were harvested by centrifugation at 8,000 x *g* and 4 °C for 15 min and the cell pellets were stored at −80 °C until use.

Cell pellets were resuspended in Buffer A (500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 g of wet pellet in 10 ml) and disrupted by sonication (QSonica Misonix 200, Amp 40, with 1 s pulse and 5 s pause for 2 min total pulse). 1 mM $CuCl_2$ was then added to the cell lysate immediately after sonication and the mixture was incubated for 1 hr at 4 °C with gentle rocking. Whole cell lysate was centrifuged at 16,000 x *g* for 15 min and the soluble fraction was harvested and applied on a 2 ml gravity Ni-NTA column (Qiagen, Valencia, CA) previously equilibrated with Buffer A. The column was subsequently washed thoroughly with Buffer A and then Buffer A containing 20 mM imidazole and the target protein was eluted in Buffer A supplemented with 150 mM imidazole. To remove imidazole from the protein samples, purified proteins were buffer-exchanged into Buffer A or Buffer B (500 mM NaCl, 50 mM NaPOi, pH 8.0) via 30-kDa ultrafiltration spin columns (Amicon Ultra, Millipore, Billerica, MA), as appropriate, concentrated to 20-70 mg/mL using the same column, and stored at −80 °C until use. The concentrations of purified proteins were determined by measurement of absorbance at 280 nm using a NanoDrop 1000 (Thermo Fisher Scientific). For sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis (PAGE) analysis, appropriately diluted protein samples were mixed with 2× SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 20% glycerol, 10% w/v SDS, 0.1% w/v bromophenol blue, 4% β-mercaptoethanol), and the samples were incubated at 95 °C for 5 min prior to loading onto gels. The gels were stained with Coomassie Brilliant Blue R250 for visualization.

Fluorescent Labeling

Dibenzocyclooctyne-PEG4-Fluor 545 (DBCO-PEG4-TAMRA, compound **1**) was dissolved in DMSO to 10 mM and the purified mutant SH3-SLAC$_{AzF}$ or WT SH3-SLAC were diluted in Buffer B to 5 µM. One point two µL of DBCO-PEG4-TAMRA (final 400 µM) or solvent control (DMSO) was mixed with 28.8 µL of SLAC solution. The molar ratio of protein to DBCO-PEG4-TAMRA is 1:80. The reaction was incubated at room temperature (RT) in the dark overnight, and the samples were loaded on an SDS-PAGE gel which was imaged under Trans UV light then stained with Coomassie Blue and imaged again under white light.

Enzymatic Assays

The enzymatic assay of each purified SH3-Slac was carried out in Buffer C (50 mM NaPOi, pH 6.0, 0.1% NaN_3) at RT. Each reaction contained 100, 20, 10, 5 or 1 mM 2,6-dimethoxyphenol (DMP) as the substrate[1] and 0.44 µM of the enzyme. Product formation was monitored by measurement of the absorbance at 468 nm (ε = 14,800 M^{-1} cm^{-1} for the dimeric product)[4] every 30 sec for the initial 10 min in a SpectraMax 340PC384 plate reader (Molecular Devices, Sunnyvale, CA). The initial highest rate within 1.5 - 2.5 min was used to determine the substrate-dependent reaction velocity, which was then fit with the Lineweaver-Burk double reciprocal plot ($1/V = 1/V_{max} + (K_M/V_{max})\ (1/[S])$) of the Michaelis-Menten equation ($V = V_{max}\ [S]/\ ([S] + Km)$) to calculate the K_M and V_{max}. The catalytic constant K_{cat} was determined as $V_{max}/\ [E]$ (Figure 3).

Based on the above calculations, 100 mM DMP was a substrate saturating concentration, and this concentration was used to determine the V_{max} and actively immobilized [E] of enzyme-immobilized electrodes in the enzymatic assay (Table 1). Product formation was monitored every 3 min for the initial 18 min using the UV-Vis module of the NanoDrop 1000. The initial highest rate within the first 6 min was used to calculate the V_{max}:

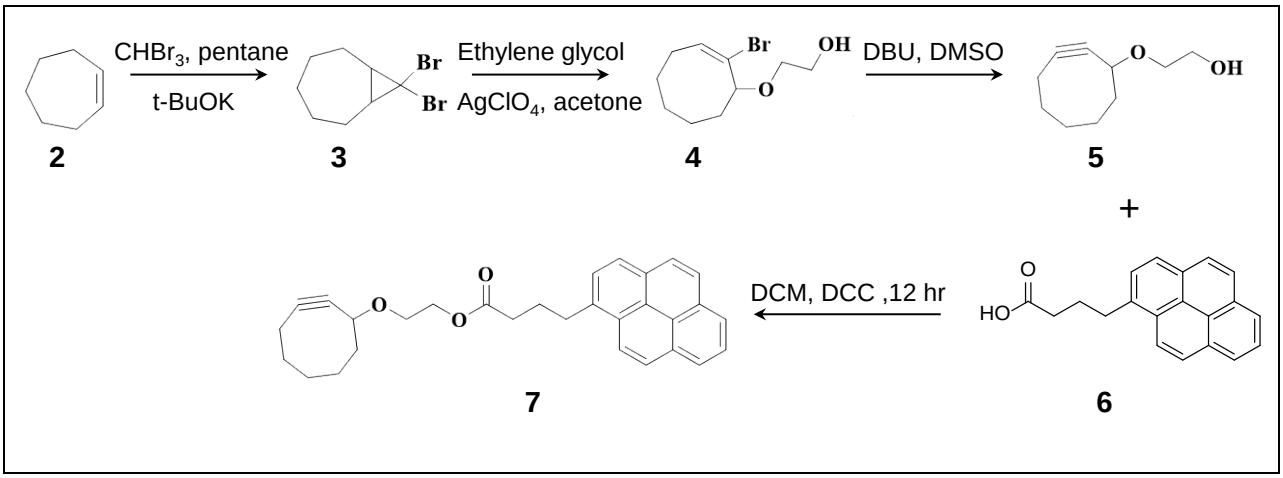
$$2DMP + O_2 \xrightarrow{Slac} 3,3',5,5'\text{-tetramethoxydiphenylquinone} + 2H_2O$$

$V_{max} = \Delta O_2/\Delta T = \Delta$dimeric product$/\Delta T = \Delta$[dimeric product] x Vol.$/\Delta T = \Delta A468$ x Vol.$/(\varepsilon$ x length of light path x $\Delta T)$

Vol.: 0.4 ml; ε: 14,800 M^{-1} cm^{-1}; length of light path: 0.1 cm; ΔT: 360 sec

Synthesis of PBCO

Scheme S1.



2.0 g cycloheptene (20.5 mmol, compound **2**), 4.66 g potassium tert-butoxide (t-BuOK, 41.5 mmol), and 10 mL anhydrous pentane were mixed in a dry round-bottom flask under argon. The mixture was vigorously stirred in an ice/salt bath (<-5 °C), and 2.25 ml bromoform ($CHBr_3$, 30.0 mmol) were added dropwise. Once the addition was complete, the mixture was vigorously stirred under argon overnight at ambient temperature. The next day, approximately 50 mL of water was added to the mixture and the pH was neutralized with 1 M HCl. The organic and aqueous phases were separated in two separate flasks. The aqueous phase was extracted 3 times with 20 mL pentane and the combined organic layer was washed with 1 M HCl/saturated NaCl solution (50 mL each) 3 times and dried over Na_2SO_4. After drying, the solvent was evaporated under vacuum to give an orange-yellow oil, which was subsequently purified by filtration through silica with n-hexane/ethyl acetate (ratio: 95/5) as eluent. A colorless oil of compound **3** was obtained with a total weight of 3.90 g and 70% yield.

3.0 g compound **3** (11.15 mmol) and 11.1 ml anhydrous ethylene glycol (223.5 mmol) were dissolved in anhydrous acetone (18 ml), and 6.95 g anhydrous $AgClO_4$ (33.55 mmol) was added in small portions under exclusion of light and stirred at room temperature for 1 hr. After addition of ethyl acetate (60 ml) and filtration, 1 M HCl (60 ml) was added and the aqueous layer was extracted with 50 ml ethyl acetate for 3 times. The combined organic layer was washed with 1 M HCl/saturated NaCl solution (100 ml each) and dried over Na_2SO_4. The solvent was evaporated under reduced pressure and compound **4** was obtained as a yellow oil with a total weight of 1.5 g and 38% yield and subsequently used without further purification.

1.5 g compound **4** (6.0 mmol) was dissolved in 20 ml DMSO and heated to 60 °C. 1.83 mL 1,8-Diazabicycloundec-7-ene (DBU, 12.0 mmol) was added, the resulting solution was stirred for 15 min and another 7.3 ml DBU (48.0 mmol) was added. The mixture was stirred at 60 °C overnight and then cooled to ambient temperature. Ethyl acetate (100 mL) and water (100 mL) were then added. After acidification to pH 1 with concentrated HCl, the aqueous phase was extracted with 50 ml ethyl acetate 3 times. The combined organic layer wase washed with 1 M HCl/saturated NaCl solution (100 ml each), dried over Na_2SO_4 and evaporated under reduced pressure. Subsequent purification by filtration through silica with n-hexane/ethyl acetate (95/5) as eluent afforded a light yellow oil of compound **5** with a total weight of 0.6 g and 60% yield.

0.15 g compound **5** (0.89 mmol) and 0.308 g 1-pyrenebutyric acid (compound **6**, 1.07 mmol) were dissolved in anhydrous dichloromethane (DCM, 18 ml), 0.275 g N,N'-dicyclohexylcarbodiimide (DCC, 1.33 mmol) dissolved in DCM was added dropwise at 0 °C under argon and stirred at room temperature for 12 hr. The formed dicyclohexylurea was filtered off and the solvent was evaporated under reduced pressure. The described silica filtration finally produced cyclooctynyloxyethyl 1-pyrenebutyrate (PBCO, compound **7**) with a total weight of 0.22 g and 56% yield as a white solid.

Electrode Preparation

Buckypaper was cut into small pieces of 5 mm x 5 mm and adhered to ITO electrodes with silver conductive epoxy glue. The buckypaper-containing electrodes were kept at RT overnight to allow the glue to fully cure, then incubated with PBCO or PBSE (each 10 mM in DMSO) with gentle rocking for 1 hr at RT, thoroughly rinsed with DMSO to remove excess PBCO or PBSE and then washed in Buffer D (50 mM NaPOi, pH 7.0) to remove all organic solvent. Purified SH3-SLAC was diluted in Buffer D to obtain the desired concentrations. The PBCO or PBSE-buckypaper-functionalized electrodes were then incubated in the appropriate protein solution with gentle rocking overnight at RT. The next day, the enzyme-modified electrodes were rinsed with Buffer D and used immediately for enzymatic and electrochemical experiments.

Enzymatic measurement of laccase-functionalized electrode

The amount of total enzymes immobilized on the electrode were estimated from the amount of enzymes remaining in the solution. The amount of active enzyme on each electrode were determined using the same DMP substrated. Briefly, each SLAC-modified buckypaper-ITO electrode was immersed in 0.4 mL O_2 saturated Buffer C (50 mM NaPOi, pH 6.0, 0.1% NaN_3) containing 100 mM DMP. An aliquot was removed every 3 min for the first 18 min and the amount of product formation was quantified by absorbance at 468

Electrochemical Measurements

All electrochemical experiments were carried out in Buffer C at RT in a conventional three-electrode cell using a WaveNow USB potentiostat (Pine Research Instrumentation, Durham, NC). A platinum wire (CH Instruments, Austin, TX) and an Ag/AgCl (saturated KCl) reference electrode (Pine Research Instrumentation) were used as the counter and reference electrodes, respectively. The chronoamperometric (CA) response was recorded at 0.2 V vs SHE with N_2 or O_2 sparging through the solution. O_2-responsive current was calculated by subtracting background N_2 saturation current from plateau O_2 saturation current. To calculate catalytic current generated by enzyme ($I_{catalytic}$), the O_2-responsive current of the same electrode before enzyme immobilization, if any, was subtracted from that following enzyme immobilization. The V_{max} of DET was calculated as follows:

$$O_2 + 4H^+ + 4e^- \xrightarrow{\text{Slac}} 2H_2O$$

$V_{max} = \Delta O_2/\Delta T = \Delta e^-/\Delta T/4 = \Delta$coulomb of charge x 6.24 x $10^{18}/\Delta T/4 = I_{catalytic}$ x 6.24 x $10^{18}/4$

Supplementary Figures

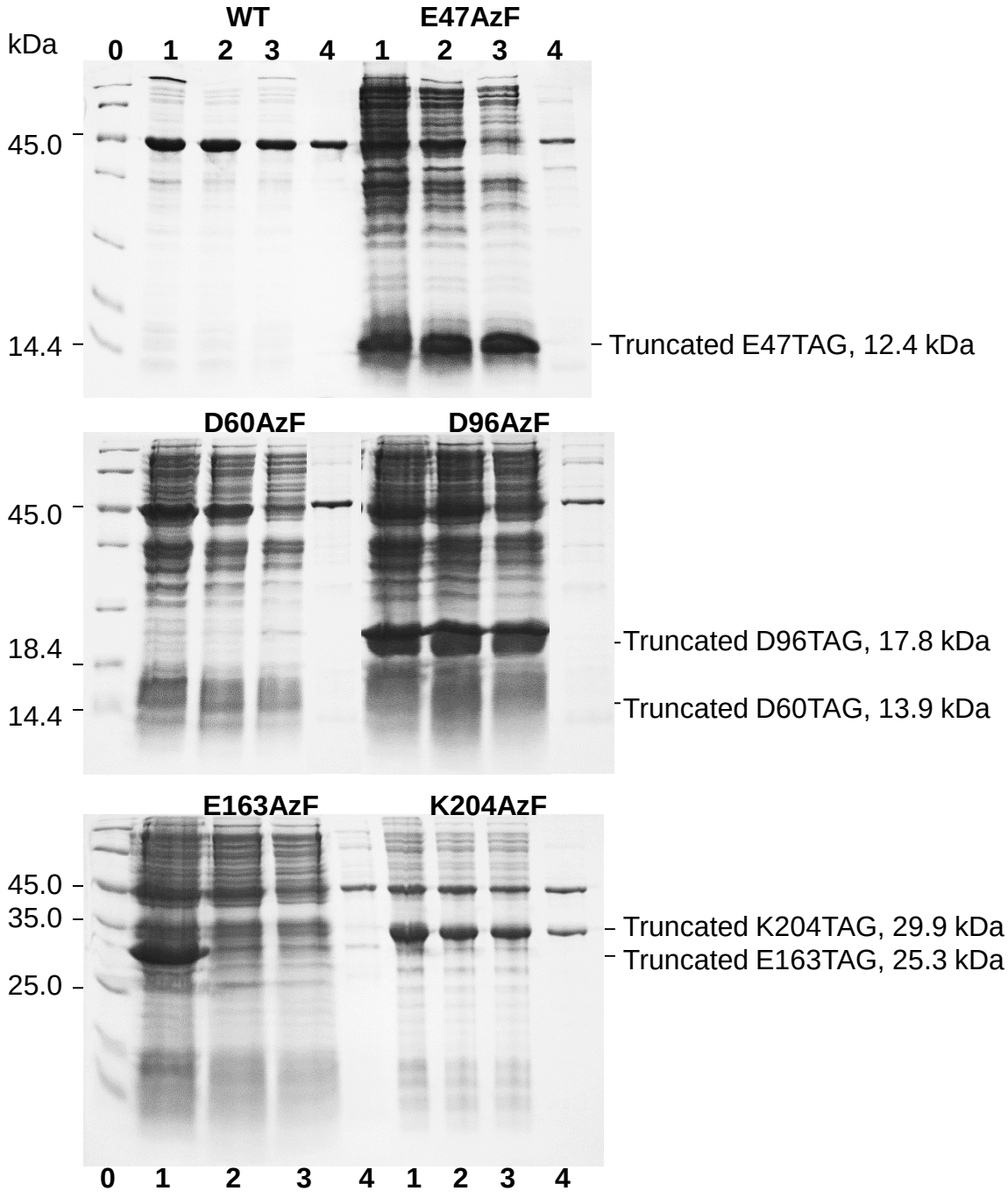


Figure S1. IMAC purification of SH3-SLAC$_{AzF}$ and WT. Lane 0: EZ-Run protein maker; lane 1: total cell lysate; lane 2: soluble lysate; lane 3: flow through; lane 4: elution. The size of the full-length protein is 45.9 kDa.

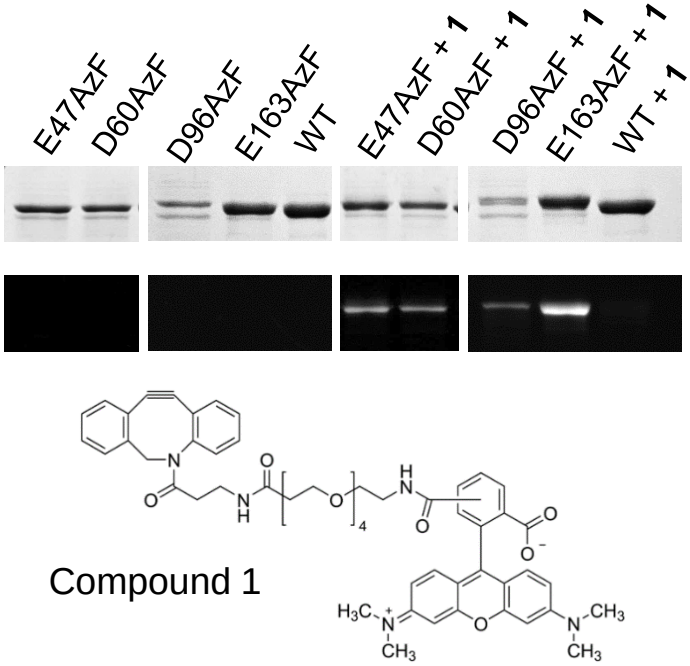


Figure S2. DBCO-PEG4-TAMRA (compound 1) specifically incorporates onto AzF-containing SLAC variants. Top panel: purified proteins stained with Coomassie Blue in 12% SDS-PAGE gels exposed to trans white light; bottom panel: imaging of the same gels under trans UV light.

	K_{cat} (sec^{-1})	$K_{M, app}$ (mM)	V_{max} (10^{-4}mM · sec^{-1})
SH3-SLAC E47AzF	0.56 ± 0.06	10.79 ± 0.04	2.47 ± 0.27
SH3-SLAC D60AzF	0.79 ± 0.10	9.79 ± 0.21	3.49 ± 0.44
SH3-SLAC D96AzF	0.39 ± 0.02	12.03 ± 0.11	1.69 ± 0.07
SH3-SLAC E163AzF	1.01 ± 0.03	8.56 ± 0.41	4.46 ± 0.15
SH3-SLAC WT	0.98 ± 0.05	8.74 ± 0.28	4.30 ± 0.22

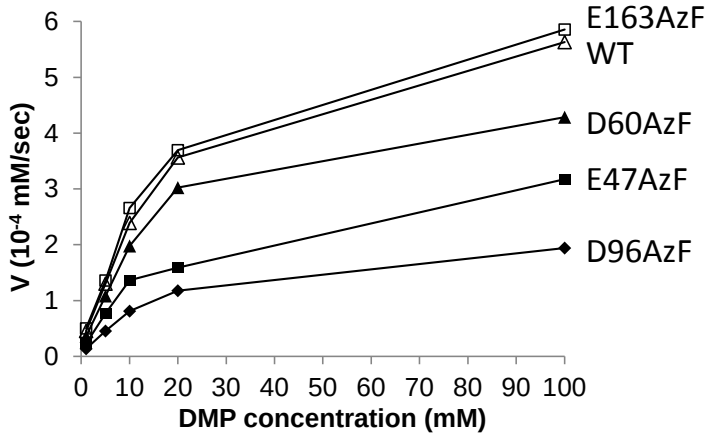
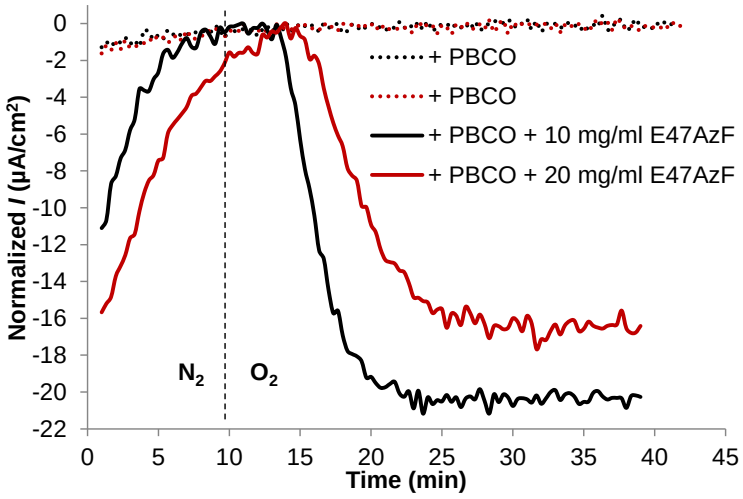


Figure S3. Kinetics of AzF-containing SH3-SLAC variants and WT SH3-SLAC at pH 6.0. Data from two independent experiments were used to calculate the errors shown in the table and representative data from one of the two experiments is presented in the chart.

A



B

	Immobilized enzyme (nmol)	Actively immobilized enzyme (10^{-2} nmol)	Catalytic current density (μA/cm²)	V_{max} for DET (10^{-2} nmol/sec)	V_{max} for DMP (10^{-2} nmol/sec)	Electron transfer efficiency (%)
10 mg/ml	1.46 ± 0.28	8.41 ± 0.07	-20.88 ± 0.42	1.35 ± 0.03	6.64 ± 0.05	20.39 ± 0.55
20 mg/ml	2.27 ± 0.08	11.5 ± 0	-18.61 ± 1.33	1.21 ± 0.09	9.08 ± 0	13.30 ± 0.95

Figure S4. (**A**) Effect of incubated enzyme concentration on DET electrode performance determined by chronoamperometric measurement of DET current at 0.2 V vs. SHE. PBCO-modified electrodes were incubated overnight with 10 or 20 mg/mL E47AzF SH3-SLAC. For each measurement, the background current density after N_2 saturation was normalized to be 0. This experiment was carried out with duplicate electrodes and representative data from one of the electrodes from each enzyme incubation is presented here. (**B**) V_{max} for PBCO-modified electrode incubated overnight with different concentrations of E47AzF SH3-SLAC. The electron transfer efficiency was calculated as the ratio between the V_{max} values for DET and DMP. Error bars represent the standard deviation of duplicate electrodes.

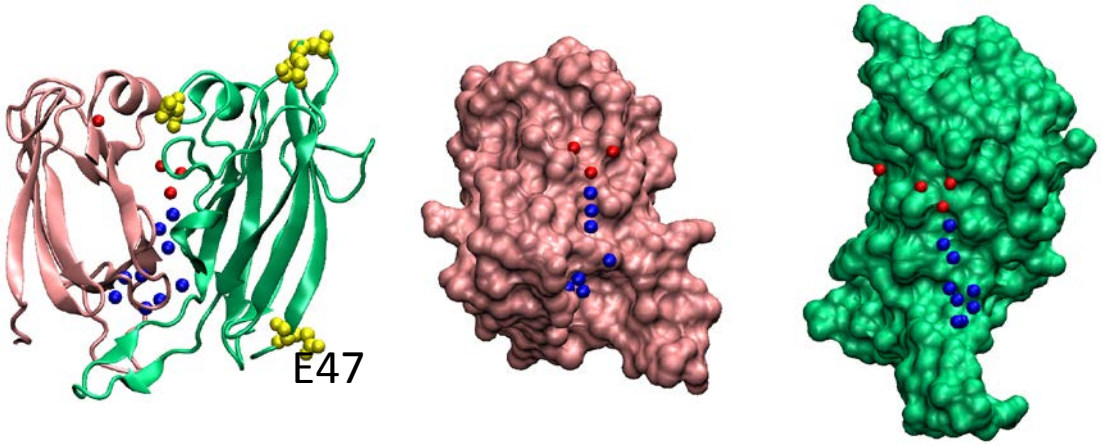


Figure S5. (left) Cartoon illustration of the water channel between two SLAC monomer. Only domains adjacent to the interface are included for clarity. (middle, right) Surface illustration of the interface of the two SLAC monomers. Blue sphere: water molecule; red sphere: catalytic copper ions; yellow residues: candidate sites for AzF incorporation.

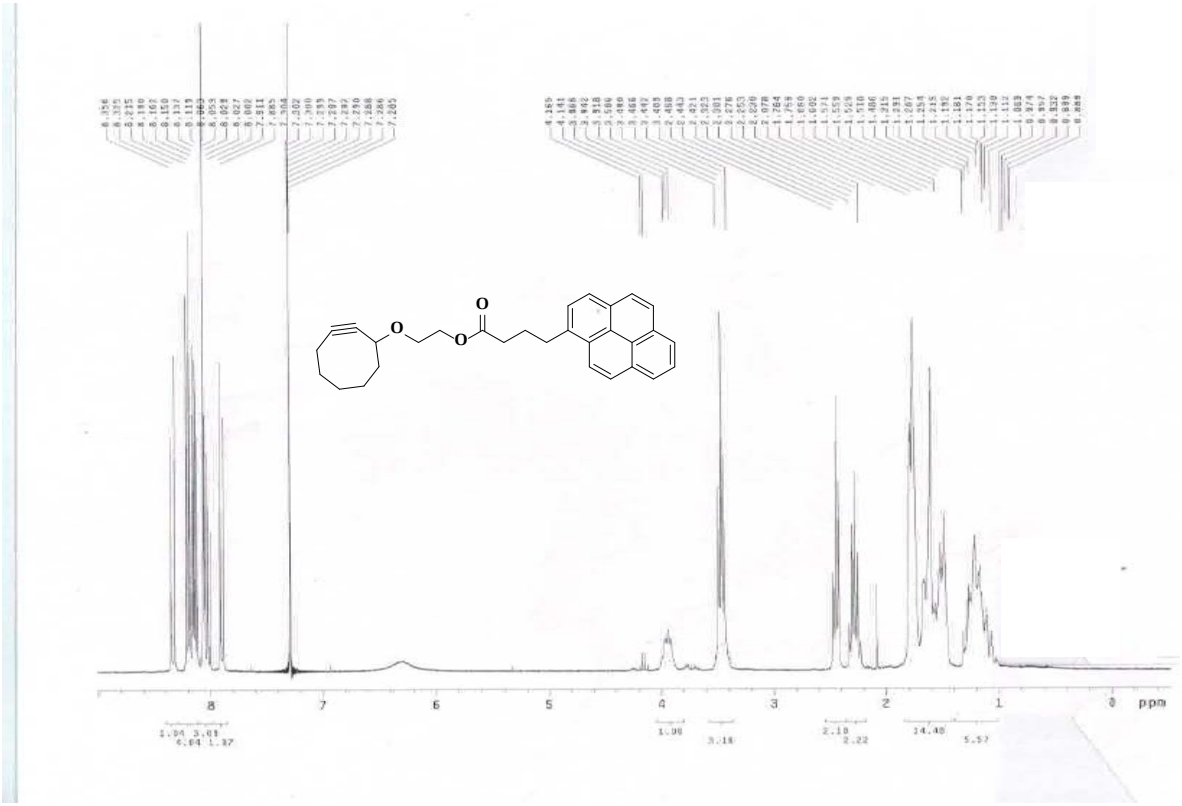
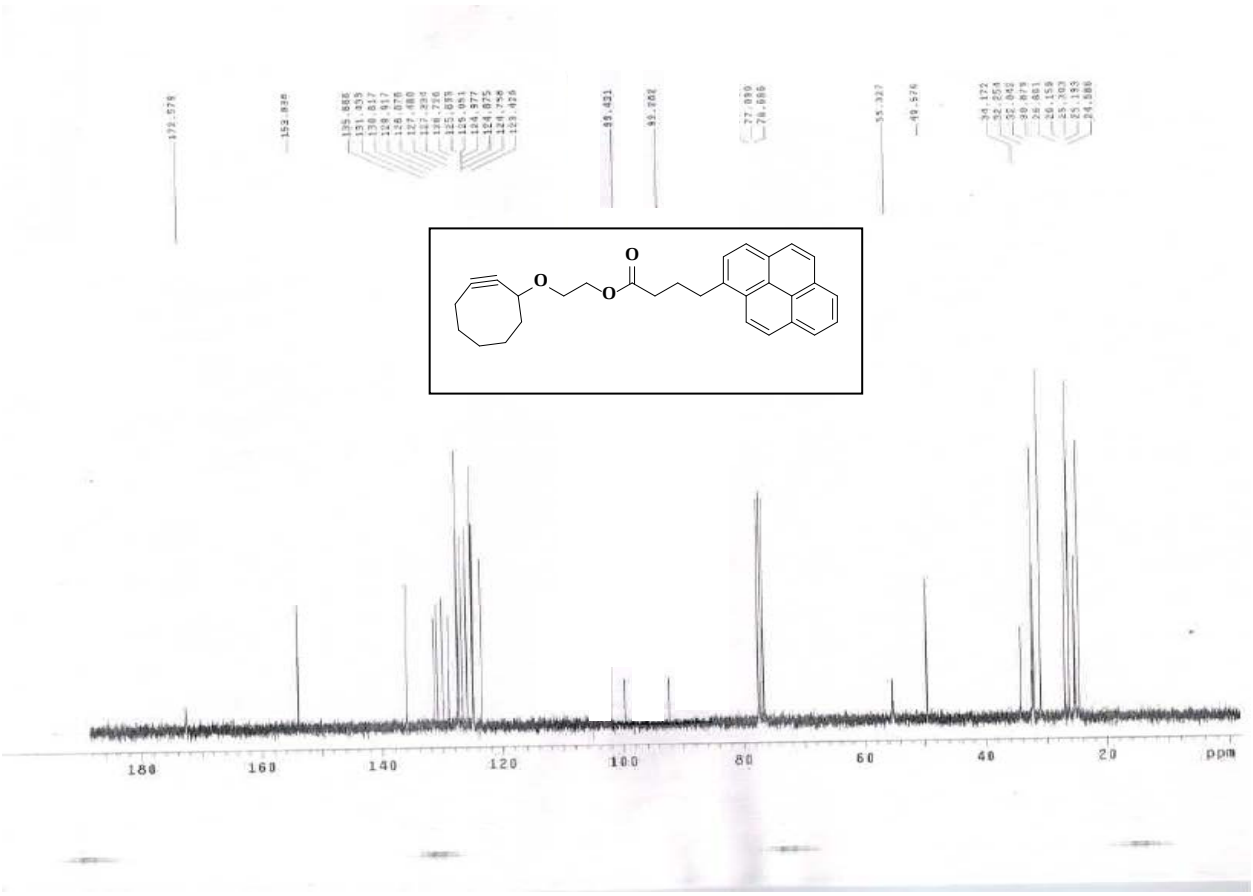


Figure S6. ^{1}H NMR spectrum of synthesized and purified PBCO. ^{1}H NMR of compound **7**; ^{1}H NMR (CD_3Cl_3, 300 MHz) δ 8.34 (d, 1H *J* = 9.3 Hz), 8.21-8.13 (m, 4H), 8.05-8.11 (m, 3H), 7.91 (d, 1H *J* = 7.8 Hz), 3.95-4.11(m, 1H), 4.11-5.82 (m, 4H), 2.42 (t, 2H, *J* = 6.0 Hz), 2.22-2.38(m, 2H), 1-68-1.82(m, 4 H), 1-42-1.65(m, 4 H), 1.28-1.06(m, 4H).



^{13}C NMR spectrum of synthesized and purified PBCO

Table S1. Primers used in this study (bases corresponding to the restriction sites and the amber stop codon are in italics and bold font, respectively).

Name	5' to 3' sequence
Slac-F	ATAA*GCTAGC*ATGGACAGGCGAGGC
Slac-R	CATT*CTCGAG*GTGCTCGTGTTCGTG
Slac E47TAG-F	GCACCGGCCGGGGGC**TAG**GTGAGACACCTCAAG
Slac E47TAG-R	CTTGAGGTGTCTCAC**CTA**GCCCCCGGCCGGTGC
Slac D60TAG-F	GCCGAGAAGCTGGCG**TAG**GGTCAGATGGGCTAC
Slac D60TAG-R	GTAGCCCATCTGACC**CTA**CGCCAGCTTCTCGGC
Slac D96TAG-F	TTCACCAACACGATG**TAG**GTGCGGGCCAGCCTG
Slac D96TAG-R	CAGGCTGGCCCGCAC**CTA**CATCGTGTTGGTGAA
Slac E163TAG-F	CACGTCGTCGGCACC**TAG**CACGGCACCGGAGGC
Slac E163TAG-R	GCCTCCGGTGCCGTG**CTA**GGTGCCGACGACGTG
Slac K204TAG-F	ACCATCAACAACCGC**TAG**CCGCACACCGGCCCC
Slac K204TAG-R	GGGGCCGGTGTGCGG**CTA**GCGGTTGTTGATGGT

Table S2

At pH 6		**Catalytic current density ($\mu A/cm^2$)**		**% Reduction over 8 days**
SH3-SLAC E47AzF	+ PBCO	-8.85 ± 2.05	Day 0	13.56 ± 2.11
		-7.68 ± 1.96	Day 8	
	+ PBSE	-1.20 ± 0.59	Day 0	46.99 ± 14.89
		-0.68 ± 0.49	Day 8	

The catalytic current density of SLACE47AzF on PBCO-modified electrode on day 0 and day 8, and percentage of current reduction over 8 days is indicated. Error rates represent the standard deviation of duplicate electrodes.

Table S3. Distances of different AzF mutants to the T1 copper and the nearest copper in the trinuclear copper site, according to the crystal structure. Pdb code: 3CG8

	Distance to T1 (Å)	Distance to trinuclear site (Å)	Electron transfer efficiency of PBCO electrode (%)
E47AzF	37	25	28.7
D60AzF	22	24	7.45
D96AzF	31	21	4.12
E163AzF	16	9	1.89

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