Supporting Information

for

Phosphopantetheinyl Transferase Catalyzed Site-Specific Protein Labeling with ADP Conjugated Chemical Probes

Yekui Zou and Jun Yin

Department of Chemistry, University of Chicago, 929 E. 57th Street, GCIS E505A, Chicago, IL 60637

Supplementary Figures

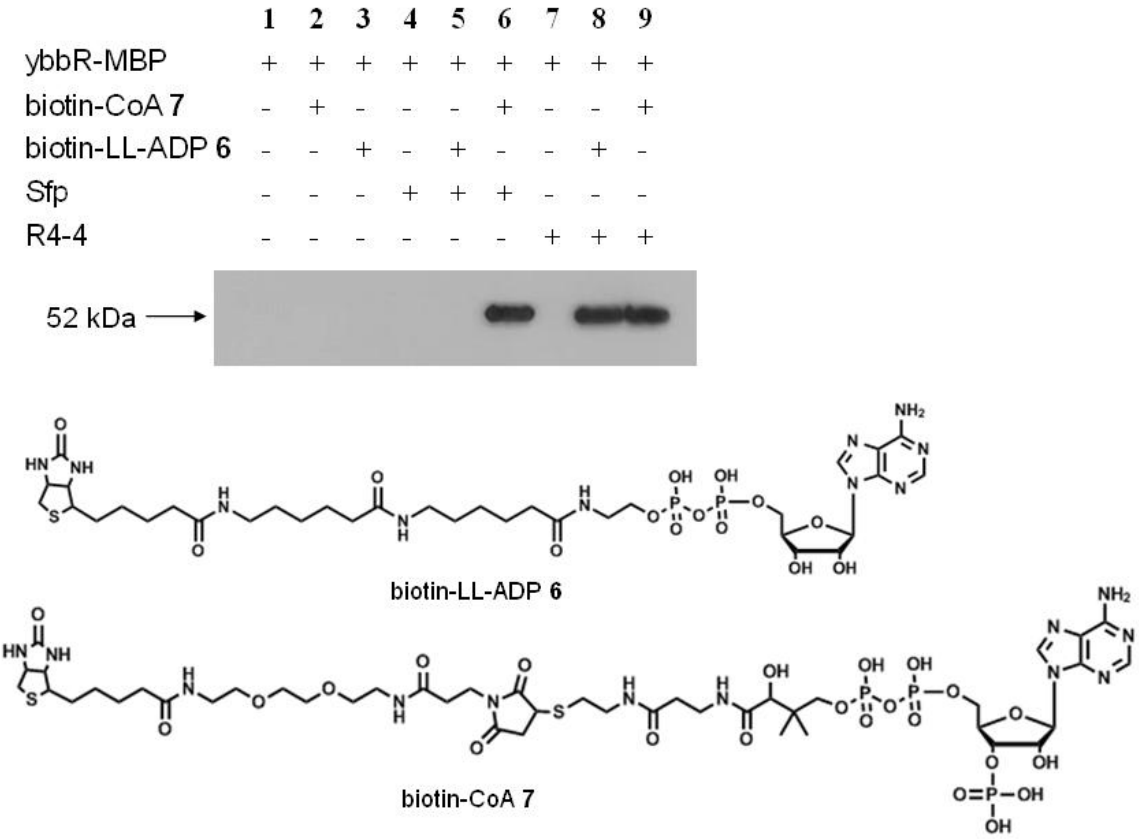


Figure S1. Site-specific labeling of ybbR-MBP fusion protein with biotin-LL-ADP **6**. The ybbR tag is fused to the C-terminus of MBP. Sfp catalyzed protein labeling with biotin conjugated to native CoA (biotin-CoA **7**) was used as a positive control.

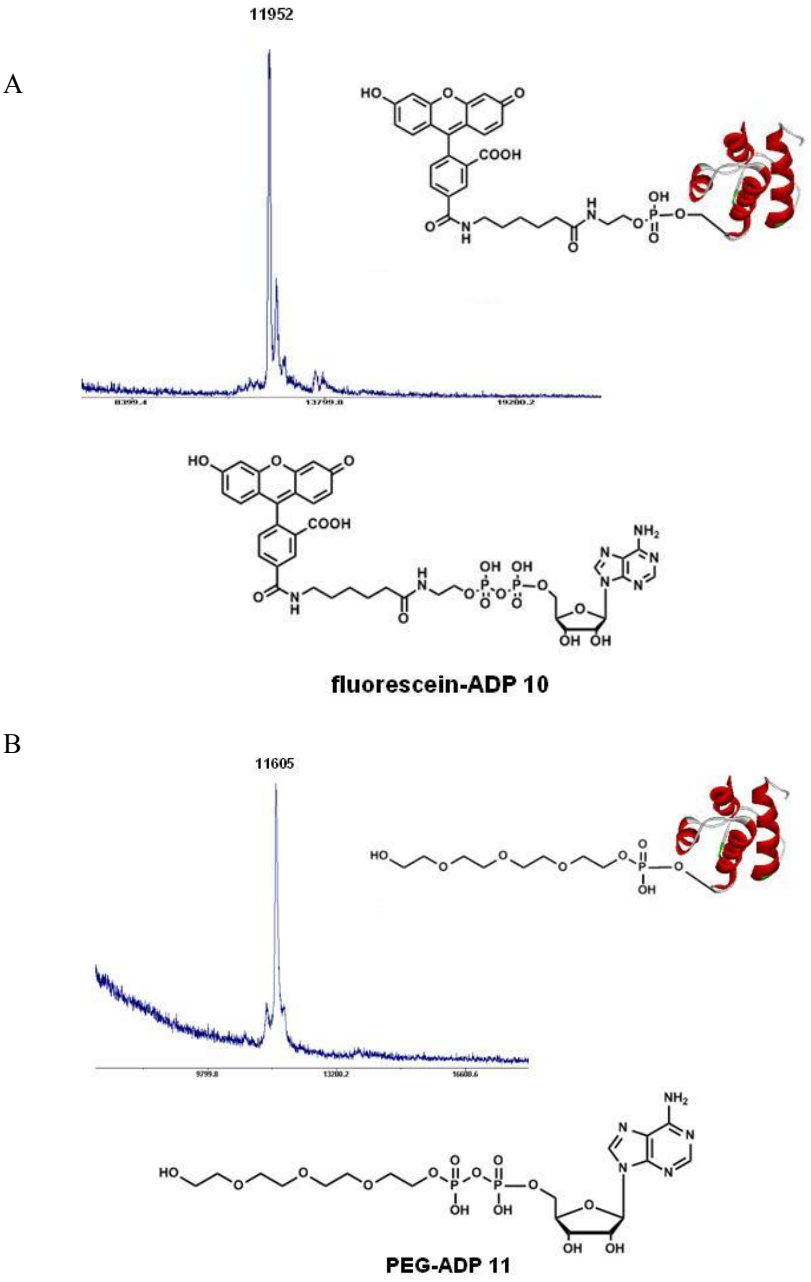


Figure S2. MALDI-TOF spectra of R4-4 catalyzed PCP modification. (A) PCP modified with fluorescein-ADP **10.** (B) PCP modified with PEG-ADP **11.**

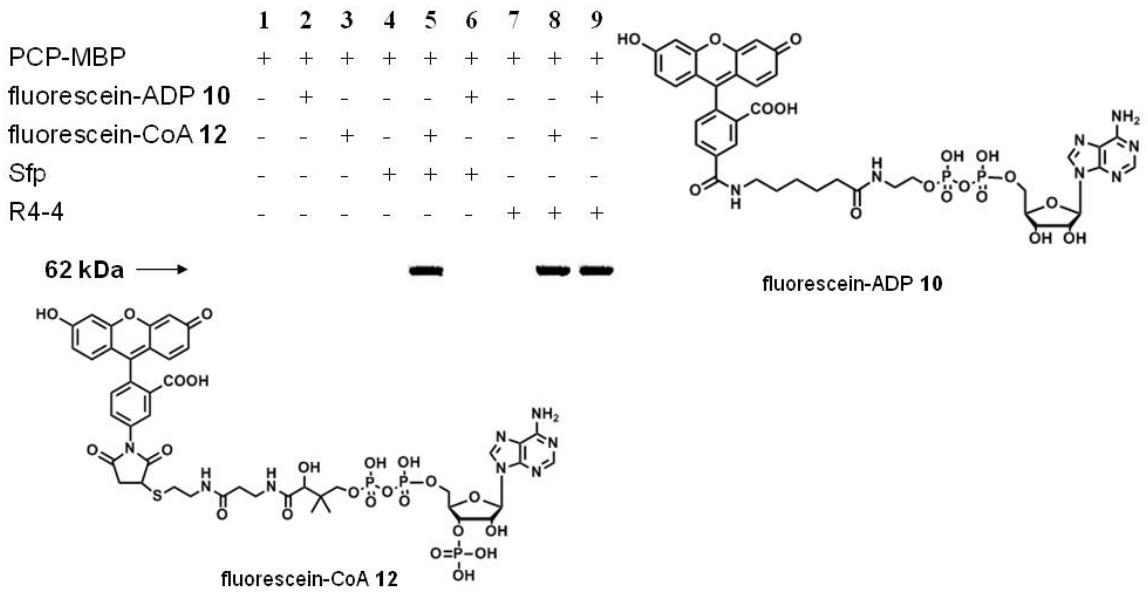


Figure S3. Site-specific labeling of PCP-MBP fusion proteins with fluorescein-ADP **10**. Protein labeling by fluorescein was visulized by florescence imaging of the SDS-PAGE gel. Fluorescein conjugated with native CoA (fluorescein-CoA **12**) was used as a positive control.

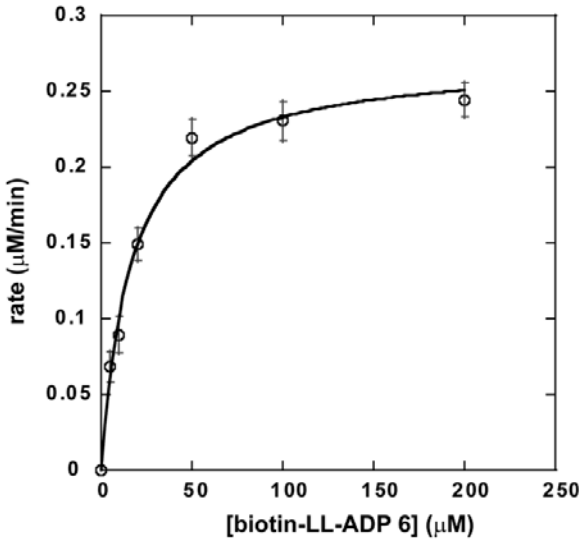
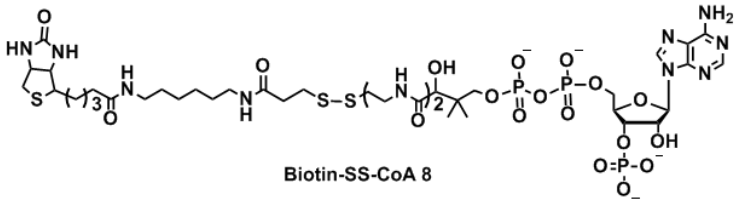


Figure S4. Kinetic analysis of R4-4 catalyzed PCP modification with biotin-LL-ADP **6**. The rate of PCP modification was measured by HPLC under saturation concentration of PCP with varying concentrations of biotin-LL-ADP **6**. The data were fit to Michaelis-Menten plot.

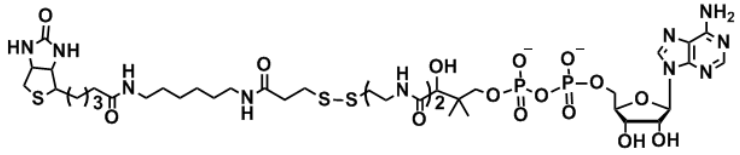
Table S1. Comparison of the kinetic parameters for PCP modification with CoA analogues catalyzed by Sfp and R4-4 under saturation concentration of PCP.

	Sfp			R4-4		
	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} min^{-1})
Biotin-LL-ADP **6**[a]	ND	ND	ND	0.136 ± 0.011	16.5 ± 1.5	0.00825
Biotin-SS-CoA **8**[b]	10.6	37.2	0.285	132	47.4	2.78
Biotin-SS-dpCoA **9**[b]	ND	ND	0.010	137	41.6	3.29

Reactions were carried out under saturation concentration of PCP at 20 μM with varying concentrations of biotin-LL-ADP **6**.
ND, not determined due to the low activity of the substrate.
[a]From this study; [b]From reference 1.



Biotin-SS-CoA 8



Biotin-SS-dpCoA 9

Experimental Section

General Unless otherwise indicated, all reagents were obtained from commercial sources and used without further purification, and all reactions were performed under nitrogen. Anhydrous solvents were purchased from Acros. All other solvents were obtained either from Fisher Scientific or from VWR International (West Chester, USA). Column chromatography was carried out on silica gel 60 (EMD Chemicals, Cincinnati, USA) with elution solvents as described. ^{1}H NMR spectra were obtained with a Bruker Model DRX 400 NMR spectrometer (Bruker, Fallanden, Switzerland). MALDI-TOF spectra were acquired with a Voyager DE PRO MALDI mass spectrometer (PerSpective Biosystems, Foster City, USA). 2,5-Dihydroxybenzoic acid was used as the matrix for small-molecule samples, and sinapinic acid was used as the matrix for protein samples, unless otherwise indicated. HPLC purification was carried out on a POLARIS BioInert Gradient LC System (Varian, Walnut Creek, USA) with a reversed-phase Nucleodur C-18 column of 250 mm in length, 21 mm ID, and 10 mm particle size (Phenomenex, Torrance, USA).

Synthesis of biotin-LL-ADP 6 *O*-phosphorylethanolamine (14 mg, 0.1 mmol) was made anhydrous by repeated dissolution in dry pyridine and evaporation of solvent (3 x 10 mL). After each evaporation step, dry N_2 was flushed into the rotary evaporator. AMP-morpholidate (4'-morpholine-*N, N*'-dicyclohexylcarboxamidinium salt) (106 mg, 0.15 mmol) was dissolved in dry pyridine and evaporated to dryness. The same process was repeated three times with exclusion of moisture under dry N_2. Both components were finally dissolved in dry pyridine and combined in a round-bottom flask under dry N_2. The reaction mixture was repeatedly evaporated from pyridine (3 x 30 mL) and flushed with dry N_2. The resulting mixture was dissolved in dry pyridine (30 mL), followed by concentration to remove about 20 mL of pyridine and the reaction

vessel was sealed under dry N_2. The solution was vigorously stirred for 48 hours. The reaction was stopped by evaporation of pyridine. The crude reaction product of adenosine 5'-diphosphoethanolamine was dissolved in water (10 mL) and purified by HPLC (anion exchange column) with a gradient of 0.01-0.40 M TEAB buffer (pH = 8.0) over 60 min at a flow rate of 2 $mLmin^{-1}$. The purified compound adenosine 5'-diphosphoethanolamine was lyophilized, and its identity was confirmed by MS (APCI): $[M+H]^+$: calcd for $C_{12}H_{21}N_6O_{10}P_2$: 471.1; found: 471.1, ^1H NMR ([D_6]DMSO): δ = 8.54 (s, 1H), 8.21 (s, 1H), 6.09 (d, *J* = 6 Hz, 1H), 4.71 (t, *J* = 4 Hz, 1H), 4.36-4.45 (m, 1H), 4.27-4.29 (m, 1H), 4.09-4.18 (m, 1H), 3.84-3.87 (m, 2H), 3.84-3.87 (m, 2H).

The product of the preceding reaction, adenosine 5'-diphosphoethanolamine (4.7 mg, 10 μmol), was added to a solution of *N*-hydroxysuccinimidyl-6'-(biotinamido)-6-hexanamido hexanoate (NHS-LC-LC-biotin, Pierce) (6.3 mg, 11 μmol) in DMF (500 μL), followed by the addition of *N*,*N*-diisopropylethylamine (3.9 mg, 30 μmol). The reaction was allowed to proceed at room temperature with stirring overnight. The reaction mixture was then purified by HPLC with a gradient of 0–65% acetonitrile in 0.1% TFA/water over 35 min at a flow rate of 10 $mLmin^{-1}$. The purified compound was lyophilized, and its identity was confirmed by MALDI-TOF (positive mode): calcd for $C_{34}H_{57}N_{10}O_{14}P_2S$: 923.33; found: 923.54. Biotin-CoA **7** was synthesized as previously reported. [2]

Synthesis of fluorescein-ADP 10 Adenosine 5'-diphosphoethanolamine (1.4 mg, 3.0 μmol) was added to a solution of 6-(fluorescein-5-carboxamido)hexanoic acid, succinimidyl ester (F6106, Invitrogen) (1.9 mg, 3.3 μmol) in DMF (50 μL), followed by the addition of *N*,*N*-diisopropylethylamine (1.2 mg, 9.0 μmol). The reaction was allowed to proceed at room temperature with stirring overnight. The reaction mixture was then purified by HPLC with a gradient of 0–65% acetonitrile in 0.1% TFA/water over 35 min at a flow rate of 10 $mLmin^{-1}$. The purified compound was lyophilized, and its identity was confirmed by MALDI-TOF (positive mode): calcd for $C_{39}H_{42}N_7O_{17}P_2$: 942.21; found: 942.81. Fluorescein-CoA **12** was synthesized as previously reported. [3]

Synthesis of PEG-ADP 11 Tetraethylene glycol monophosphate (27 mg, 0.1 mmol) was made anhydrous by repeated dissolution in dry pyridine and evaporation of solvent (3 x 10 mL). After each evaporation step, dry N_2 was flushed into the rotary evaporator. AMP-morpholidate (4'-morpholine-*N*,*N*'-dicyclohexylcarboxamidinium salt) (106 mg, 0.15 mmol) was dissolved in dry pyridine and evaporated to dryness. The same process was repeated three times with exclusion of moisture under dry N_2. Both components were finally dissolved in dry pyridine and combined in a round-bottom flask under dry N_2. The reaction mixture was repeatedly evaporated from pyridine (3 x 30 mL) and flushed with dry N_2. The resulting mixture was dissolved in dry pyridine (30 mL), followed by concentration to remove about 20 mL of pyridine and the reaction vessel was sealed under dry N_2. The solution was vigorously stirred for 48 hours. The reaction was stopped by evaporation of pyridine. The crude reaction products were dissolved in water (10 mL) and purified by HPLC (anion exchange column) with a gradient of 0.01-0.40 M TEAB buffer (pH = 8.0) over 60 min at a flow rate of 2 $mLmin^{-1}$. The purified compound was lyophilized, and its identity was confirmed by MALDI-TOF (positive mode): calcd for $C_{18}H_{32}N_5O_{14}P_2$: 604.14; found: 604.56.

Protein expression and purification pET plasmids (Novagen, Darmstadt, Germany) of pT2 were kindly provided by Professor Christopher T. Walsh at Harvard Medical School for the expression of GrsA PCP. [4] Expression of the proteins followed reported protocols. Briefly, plasmids harboring the genes of the C-terminal 6×His-tagged fusion proteins were transformed into E. coli BL21- (DE3)pLysS chemically competent cells (Invitrogen). Cells were grown at 37 ^{o}C in Luria Broth (LB, 1 L) supplemented with ampicillin (100 mg/mL) to an optical density of 0.6 at 600 nm. Protein expression was then induced with the addition of isopropyl d-thiogalactopyranoside (IPTG, 1 mm) and the cell culture was grown at 15 ^{o}C overnight. The next day, the cells were harvested by centrifugation at 5000 rpm for 15 min and the cell pellets were resuspended in lysis buffer containing Tris (pH 8.0, 50 mm), NaCl (500 mm) and imidazole (5 mm) supplemented with DNAse I (1 unit/mL). The resuspended cells were disrupted with a French Press (Thermo Spectronic, Asheville, USA) with three passages at 12000 psi. Cell debris was removed by centrifugation at 16000 rpm for 30 min. The clarified cell extract was incubated with a suspension of Ni-NTA resin (Qiagen, 50 %, 1 mL) for 3 h at 4 ^{o}C in a batch-binding format. The suspension was then loaded onto a gravity column and washed with the lysis buffer (20 mL). Protein bound to the column was eluted with elution buffer (6 mL) containing Tris(pH 8.0, 50 mm), NaCl (500 mm) and imidazole (250 mm). The purity of the fractions containing the protein of interest was checked by SDS-PAGE stained by Coomassie brilliant blue. Fractions with the desired purity were pooled and dialyzed against HEPES (50 mm, pH 7.5, 2 x 1 L), NaCl (100 mm), and glycerol (10 %). Protein solutions were then aliquoted and stored at -80 ^{o}C.

The pET vector containing the gene of the C-terminal 6×His tagged Sfp mutant R4-4 was transformed into E. coli BL21(DE3)pLysS (Invitrogen) chemical competent cells. R4-4 protein was expressed following the procedure previously reported. [1]

Sfp catalyzed protein modification In a total volume of 200 μL, Sfp phosphopantetheinyl transferase (2.0 μM), biotin-CoA **7 (**100 μM), $MgCl_2$ (10 mM) in HEPES (50 mM, pH 7.5) were incubated with protein (20 μM) for 60 minutes. The same condition was used with fluorescein-CoA **12** (100 μM).

R4-4 catalyzed protein modification In a total volume of 200 μL, R4-4 (2.0 μM), biotin-LL-AD **6 (**100 μM), $MgCl_2$ (10 mM) in HEPES (50 mM, pH 7.5) were incubated with protein (20 μM) for 60 minutes. The same condition was used for protein labeling with fluorescein-ADP **10** (100 μM) and PEG-ADP **11** (100 μM).

Measurement of the kinetics of PCP modification by biotin-LL-ADP 6 The kinetic measurement for R4-4 catalyzed PCP labeling was carried out in buffer (10 mM $MgCl_2$ and 50 mM HEPES, pH 7.5) in the presence of 2 μM R4-4. The concentration of biotin-LL-ADP **6** in the reaction was varied from 2 μM to 200 μM at a constant PCP concentration of 20 μM. The reaction was allowed to proceed at 37ºC for 15 minutes and 60 minutes before quenching by the addition of 30 μL 4% TFA to 100 μL reaction mixture. The reaction was then analyzed by analytical HPLC with a reverse phase C18 column using a gradient of 30-50% CH_3CN in 0.1% TFA/H_2O over 30 minutes and monitored at 220 nm. HPLC peak areas were integrated, and the product concentration was calculated as a percentage of the total peak area. Initial velocity data were fit to the Michaelis-Menten equation by the computer software KaleidaGraph.

Western blot Labeling reaction mixture (20 μL) was loaded on a 4–15% SDS-PAGE gel (Bio-Rad). After electrophoresis, the protein bands were electroblotted onto a piece of polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The membrane was then blocked with BSA (3%) in TBS buffer for an hour, followed by incubation with BSA (1%) in TBS buffer containing $1/10^6$ diluted 1 $mgmL^{-1}$ streptavidin-HRP conjugate (Pierce) for 1 hour. The membrane was then washed with Tween 20 (0.05%) and Triton X-100 (0.05%) in TBS (6x) and with TBS (6x) followed by detection with the ECL luminescent detection kit (Amersham Pharmacia).

Fluorescence visualization Labeling reaction mixture (20 μL) was loaded on a 4–15% SDS-PAGE gel (Bio-Rad). After electrophoresis, fluorescence gel visualization was performed using a Bio-Rad Molecular Imager FX with excitation at 488 nm.

References

1. Sunbul, M.; Marshall, N. J.; Zou, Y.; Zhang, K.; Yin, J., *J Mol Biol* **2009,** *387,* 883-898.
2. Yin, J.; Liu, F.; Li, X.; Walsh, C. T., *J Am Chem Soc* **2004,** *126*, 7754-5.
3. Yin, J.; Lin, A. J.; Golan, D. E.; Walsh, C. T., *Nat Protoc* **2006,** *1*, 280-5.
4. Stachelhaus, T.; Walsh, C. T., *Biochemistry* **2000,** *39*, 5775-87.