Reversible labeling of native and fusion-protein motifs

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The reversible covalent attachment of chemical probes to proteins has long been sought as a means to visualize and manipulate proteins. Here we demonstrate the full reversibility of post-translational custom pantetheine modification of Escherichia coli acyl carrier protein for visualization and functional studies. We use this iterative enzymatic methodology in vitro to reversibly label acyl carrier protein variants and apply these tools to NMR structural studies of protein-substrate interactions.

Post-translational protein modification is important for adding functions to proteins that can be exploited for therapeutics, protein engineering, affinity design and enzyme immobilization, among other applications. Acyl carrier protein (ACP) labeling with 4-phosphopantetheine (PPant), conjugated to a tag of choice by its transferase (PPTase), represents one of the most flexible covalent protein labeling methods, as illustrated by its application in tagging minimal-ACP peptides, bio-gel formation and ACP-dependent protein immobilization. The labeling of ACP and ACP fusion proteins with PPant analogs is also successfully leveraged for visualization, isolation and functional and structural studies of carrier protein–dependent biosynthetic enzymes. Yet, further advancement of these tools is hampered by an inability to easily reverse PPant attachment. Naturally occurring ACPs, often isolated in holoform (which includes a native PPant modification), cannot be further modified directly with another PPant tag. To overcome these difficulties, we use ACP hydrolase (AcpH), a phosphodiesterase from Pseudomonas aeruginosa, and Sfp, a PPTase from Bacillus subtilis, to swap different PPant-conjugated small molecules on free ACP and ACP fusion proteins (Fig. 1). This reversible tagging system offers the ability to connect synthetic and biological chemistry with ease and provides uniformly labeled, high-quality ACP and ACP fusion proteins, as demonstrated here through fluorescence labeling and solution-phase protein NMR.

For evaluation of iterative labeling, we began with fluorescent ACP labeling directly in cellular lysate (Supplementary Fig. 1a) from E. coli strain DK554, which overexpresses native fatty acid ACP (AcpP) in predominantly apo form. Treatment of this lysate with coumarin-CoA and Sfp generated a blue fluorescent band upon excitation of SDS-PAGE samples at 254 nm that co-migrated with a coumarin-labeled ACP standard. Subsequent treatment of coumarin-labeled lysate with recombinant AcpH uniformly removed the coumarin-PPant from ACP, as demonstrated by the disappearance of the blue band (Supplementary Fig. 1b). Subsequent treatment of the sample with Sfp and rhodamine-CoA generated a new red fluorescent SDS-PAGE band upon excitation at 532 nm (Supplementary Fig. 1c); this label can also be removed (Fig. 2a and Supplementary Fig. 2a) with AcpH.

After demonstrating the compatibility of AcpH for removing various PPant analogs, we sought to demonstrate the flexibility of our technique by evaluating interaction with ACP fusion proteins. AcpH removed rhodamine-PPant from ACP attached to three different fusion partners: an N-terminal maltose-binding protein (MBP), a C-terminal GFP and an N-terminal bacterial luciferase (Lux-ACP) (Fig. 2b and Supplementary Figs. 2b, c, 3 and 4). The activity of the luciferase-ACP fusion did not change notably following label manipulation (Supplementary Fig. 5).

**Figure 1** | Reversible labeling of E. coli ACP. Recombinant [15N]ACP is isolated in the holo-state (top, yellow). [15N]ACP is prepared for covalent labeling by treatment with AcpH to generate exclusively apo-[15N]ACP (red). Protein purity and modification homogeneity is confirmed by two-dimensional NMR (top right). Labeling with acyl pantetheine analogs to the crypto-[15N]ACP (blue), or ‘labeled’, form proceeds via PPTase and acyl-CoA (mCoA) modification that is analyzed by NMR (bottom right, top left). Modification is quantitatively reversed by AcpH, whereby labeled proteins are returned to the apo form.

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We additionally found that labeling of the GFP-ACP fusion with rhodamine-CoA generates an observable Förster resonance energy transfer (FRET) signal (Supplementary Fig. 6), which enables observation of AcpH or PPTase activity in a simple and scalable assay format.

Recent studies of fatty acid and polyketide pathways focus on the extent and function of substrate sequestration by ACP, in which the growing acyl chain is covalently attached via a thioester linkage to the terminus of post-translationally added 4'-phosphopantetheine. Biosynthetic intermediates with varied chemical structures participate in intramolecular interactions with ACP that modulate substrate dynamics and ACP structure.

The nature of these ACP-substrate interactions depends on the chemical structure of the biosynthetic intermediate and can vary with respect to chain length and oxidation state. Furthermore, observations of this phenomenon appear to vary depending on the analytical method used. X-ray crystallography of hexanoyl-, heptanoyl- and decanoyl-ACPs from E. coli fatty acid biosynthesis all show the acyl chain clearly buried in ACP, whereas two different crystal forms of butanoyl-ACP show the acyl chain residing outside the protein with one form and inside the protein with the other. NMR studies have shown that short-chain polyketide analogs protrude into solution when appended to Streptomyces coelicolor actinorhodin ACP, whereas saturated acyl chains of 4–8 carbons associate more closely with this polyketide ACP. Variations in substrate dynamics must clearly play a role in the catalytic processivity of these synthases, and we hypothesize that the dynamics of substrate binding serves a critical function in substrate specificity.

To evaluate substrate dynamics with respect to substrate identity, it is necessary to perform multiple studies on the same protein with varying acyl substrates covalently attached. Given the labor and expense of preparing uniformly labeled, isotope-enriched proteins for NMR structural studies, we investigated the use of AcpH as a means to recycle [15N]ACP. We reversibly labeled a single sample of [15N]-enriched E. coli ACP with several acyl pantetheines with [15C] labels within the acyl chain, directly observed intramolecular interactions of the [15N]ACP with the pendant acyl chain using nuclear Overhauser effect (NOE) measurements.

We evaluated [15N]ACP at each labeling condition via gel (Fig. 2c and Supplementary Figs. 7 and 8) and NMR analysis (Supplementary Figs. 9–14). We obtained an initial apo and holo mixture following E. coli expression that required full conversion to the apo form using AcpH. Subsequent conversion to octanoyl-[15N]ACP used the chemoenzymatic synthesis of an octanoyl-CoA analog with Sfp labeling (Fig. 2c). After NMR evaluation, we converted the [15N]ACP back to the apo form with AcpH for subsequent relabeling. We acquired [15N]-[1H] heteronuclear single-quantum coherence (HSQC) spectra of all three [15N]ACP species (apo, octanoyl- and regenerated apo). Comparing apo-[15N]ACP to the octanoyl-[15N]ACP (Fig. 3a), we observed chemical shift perturbations characteristic...
of acyl chain sequestration in the hydrophobic binding pocket. Conversion from this acylated form back to the apo form by AcpH provided uniformly unlabelled apo-[13C4]ACP, as confirmed by an HSQC spectrum of the regenerated protein that identically matched that of the original (Fig. 3b). This validated the feasibility of reversible ACP labeling, as it demonstrated that the regenerated apo-[13C4]ACP is properly folded and ready for subsequent modification.

We next labeled this regenerated apo-[13C4]ACP with a [13C4]butanoyl-CoA analog (Fig. 2c), which contained 13C labels at carbons 1–4. [13C4]Butanoyl-[13C4]ACP demonstrated weaker HSQC chemical shift perturbations (Fig. 3c) than octanoyl-[13C4]ACP. Further sample treatment involved one last conversion to the apo form by AcpH followed by labeling with [8-13C]octanoyl-CoA (containing a single 13C label at carbon 8; Figs. 2c and 3d). We performed 13C-selective NOE experiments, in which we observe NMR signals for other protons within 5 Å from the 13C label, on the [13C4]butanoyl-[13C4]ACP and [8-13C]octanoyl-[13C4]ACP as a means to gain structural information about substrate-protein interactions. In collecting 13C-edited NOE spectra of the 13C-labeled acyl [13C4]ACP, we observed NOE signal for [13C4]butanoyl-[13C4]ACP, whereas [8-13C]octanoyl-[13C4]ACP produced a notable signal (Supplementary Fig. 15). This result was likely produced from spatial proximity of an aliphatic proton in a [15N]ACP side chain and the 13CH2 group in the [8-13C]octanoyl acyl chain, indicating that the longer acyl chain resides in the protein binding pocket. This finding signifies a lack of dynamic mobility and sequestration of the acyl chain. Conversely, the negative result from [13C4]butanoyl-[13C4]ACP indicates that the shorter acyl chain is notably more dynamic in solution. The X-ray crystal structure indicates two states for a tethered butanoyl substrate: one outside (evidenced by a closed hydrophobic pocket) and one sequestered within the protein. Our NMR-based finding highlights the differences of substrate-tethered ACP behavior between solution and crystalline structure and reveals that the solution-exposed acyl chain is the predominant form of [13C4]butanoyl-[13C4]ACP in solution. We conclude that analysis of ACP-substrate dynamics must necessarily be performed in the solution state.

In addition to observing the dynamics of tethered acyl substrates, these NMR studies provide a qualitative evaluation for protein quality after repeated labeling and unlabling steps. This demonstration offered an ideal testing ground for the reversible labeling method, as we used only one isotope-enriched protein sample for the entire experiment. Any protein degradation or incomplete reactivity would severely compromise the quality of resulting NMR spectra. To provide quality control, we acquired HSQC spectra of purified [13C4]ACP at each discrete step throughout the process and compared them to the spectra of the original apo-[13C4]ACP sample (Fig. 3), which revealed retention of protein integrity throughout the experiment. By tracking the ultraviolet absorbance of [13C4]ACP throughout all conversions (Supplementary Table 1), we observed a final recovery of 27% protein after five discrete enzymatic reaction steps. We further evaluated reaction efficiency for all presented reactions (Table 1 and Supplementary Figs. 16 and 17); the values demonstrate that two-step yields above 60% are feasible for most ACP constructs.

This work suggests that AcpH is capable of removing a broad variety of covalently tethered labels beyond those studied here, in addition to accommodating N- and C-terminal ACP fusion partners with ease. Given the multitude of existing opportunities for ACP labeling, particularly in work involving fusion-protein applications and natural-product biosynthetic studies, we believe that providing a reversible methodology will provide markedly improved flexibility for rapid modification of protein species. Additionally, the cost-saving measure of recovering valuable apo-ACP substrates cannot be overlooked. Because of the wide pantetheine substrate acceptance demonstrated by a combined Sfp and AcpH methodology, various fluorescent and functional tags can be exchanged on a single protein with robustness not offered by previous enzymatic methods.

**METHODS**

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
N.M.K. performed all cloning, subcloning, enzymatic reactions, imaging and protein purifications (unless otherwise stated). R.W.H. conducted all protein NMR experiments and provided resulting NMR data. R.W.H. prepared the native Sfp, MBP-CoA,D,E enzyme stocks used for ‘one-pot’ chemoenzymatic CoA analog synthesis. A.R.S. synthesized, purified and characterized all oxopantetheine probes in this work. N.M.K., R.W.H., A.R.S. and M.D.B. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Luchansky, S.J., Argade, S., Hayes, B.K. & Bertozzi, C.R. Biochemistry 43, 12358–12366 (2004).
2. Stachler, M.D., Chen, I., Ting, A.Y. & Bartlett, J.S. Mol. Ther. 16, 1467–1473 (2008).
3. Batra, G. et al. Protein Expr. Purif. 74, 99–105 (2010).
4. Gauchet, C., Labadie, G.R. & Poulter, C.D. J. Am. Chem. Soc. 128, 9274–9275 (2006).
5. Hinner, M.J. & Johnsson, K. Curr. Opin. Biotechnol. 21, 766–776 (2010).
6. Yin, J. et al. Proc. Natl. Acad. Sci. USA 102, 15815–15820 (2005).
7. Mosiewicz, K.A., Johnsson, K. & Lutolf, M.P. J. Am. Chem. Soc. 132, 5972–5974 (2010).
8. Wong, L.S., Thrilway, J. & Micklefield, J. J. Am. Chem. Soc. 130, 12456–12464 (2008).
9. Foley, T.L., Young, B.S. & Burkart, M.D. FEBS J. 276, 7134–7145 (2009).
10. Meier, J.L. et al. ACS Chem. Biol. 4, 948–957 (2009).
11. Meier, J.L., Haushalter, R.W. & Burkart, M.D. Bioorg. Med. Chem. Lett. 20, 4936–4939 (2010).
12. Haushalter, R.W. et al. ACS Chem. Biol. 6, 413–418 (2011).
13. Murugan, E., Kong, R., Sun, H., Rao, F. & Liang, Z-X. Protein Expr. Purif. 71, 132–138 (2010).
14. Quadri, L.E. et al. Biochemistry 37, 1585–1595 (1998).
15. Chan, D.I. & Vogel, H.J. Biochem. J. 430, 1–19 (2010).
16. Evans, S.E. et al. J. Mol. Biol. 389, 511–528 (2009).
17. Ploskorf, E. et al. Chem. Biol. 17, 776–785 (2010).
18. Roujeinikova, A. et al. J. Mol. Biol. 365, 135–145 (2007).
19. Roujeinikova, A. et al. Structure 10, 825–835 (2002).
20. Upadhyay, S.K. et al. J. Biol. Chem. 284, 22390–22400 (2009).
ONLINE METHODS

Determination of protein concentration, protein gels, miscellaneous. ACP concentrations were determined by UV absorbance measurements at 280 nm unless otherwise noted. Extinction coefficients were calculated using the ExPASy (http://www.expasy.ch/) ProtParam tool: E. coli free ACP = 1,490 M$^{-1}$ cm$^{-1}$, GFP-ACP = 69,000 M$^{-1}$ cm$^{-1}$, MBP-PaACP = 66,000 M$^{-1}$ cm$^{-1}$ and Lux-ACP = 85,720 M$^{-1}$ cm$^{-1}$. Non-ACP protein concentrations and fusion ACPs used in the efficiency analysis (Table 1b) were determined using the Bradford method against a BSA standard. ACP was run on 20% 2 M urea-PAGE to resolve apo, hol and crypto conversions as well as 12% SDS-PAGE to evaluate overall purity during NMR workup. ACP fusions MBP-PaACP and GFP-ACP samples were run on 15% 2 M urea-PAGE and Lux-ACP was run on 10% SDS-PAGE for fluorescence imaging experiments. Electrophoresis of fluorescent coumarin and rhodamine nonfusion E. coli ACP modifications used 10% Tris-tricine SDS-PAGE.

Gel imaging. Coomassie-stained gels were imaged on a Fluor-S 1 L M9 minimal medium supplemented with 1 g/L 15N[acetate]. GFP-ACP images were acquired before gel fixing, after which they were fixed and imaged as other gels. GFP-ACP images were acquired as.tif files, and excess white was discarded using the Auto Levels feature of Photoshop (Adobe). UV acquisition was also performed on the Fluor-S Multilimage, with short/long wave UV and a 520LP filter, and excess black was discarded using the Auto Levels feature of Photoshop (Adobe). GFP fluorescence imaging before gel fixing was performed on a UVP BioSpectrum (UVP LLC) system with a SYBR Green 515–570-nm–emission filter. Excitation was provided by transillumination from a UVP BioLite with a 420BP40 filter. GFP fluorescence images were collected as .tif files and had gray input levels adjusted using Photoshop from ‘0,1,00,255’ to ‘0,1,00,150’ to discard excess black. Rhodamine-labeled protein gels were imaged on a Typhoon (GE Healthcare) gel scanner at 50-μm resolution with a photomultiplier tube (PMT) setting of 450 and using a 532-nm (green laser) excitation and 580BP30 emission filter. Typhoon gel images were collected as .gel files, converted to .tif in ImageJ (NIH) and exported to Photoshop, with which gray input levels were adjusted from ‘0,1,00,255’ to ‘80,1,00,255’ to discard excess whites collected from the .gel file. All gels, with the exception of GFP-ACP containing gels, were fixed with 10% acetic acid, 40% methanol and 50% water for 1 h, then rinsed three times with water before UV fluorescence imaging and subsequent staining. GFP-ACP images were acquired before gel fixing, after which they were fixed and imaged as other gels.

Production of AcpH and recombinant ACP constructs. Cloning methods and primers are contained in the Supplementary Methods and Supplementary Table 2. For expression of E. coli [15N]ACP, E. coli BL21 (DE3) cells containing plasmid pET22b encoding C-terminal 6×His-tagged E. coli ACP were cultured in 1 L M9 minimal medium supplemented with 1 g/L 15N-enriched ammonium chloride and 100 µg/mL ampicillin. Culture was grown to OD$_600$ = 0.6, induced with 1 mM IPTG and was shaken 4 h at 37 °C. E. coli BL-21 (DE3) cells containing the MBP-AcpH plasmid were grown in 1 L LB, 0.2% D-glucose and 50 µg/mL kanamycin sulfate at 37 °C to OD = 0.6, induced with 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG), and shaken at 16 °C overnight. Medium was centrifuged 30 min at 2,000 r.p.m. to pellet cells. Cell pellets were stored at −20 °C overnight. AcpH-6×His construct was grown similarly without glucose. Cells were thawed on ice and suspended in lysis buffer (50 mM TrisCl, pH 8; 500 mM NaCl; and 10% glycerol) with additional ingredients 0.1 mg/mL lysozyme, 0.1 mM DTT, 5 µg/mL DNase I and 5 µg/mL RNase A and passed twice through a French pressure device at 1,000 p.s.i. Lysate was centrifuged 45 min at 10,000 r.p.m., and supernatant was incubated with amylose resin (New England Biolabs) for MBP-AcpH or with Ni-NTA (Novagen) for AcpH-6×His according to manufacturer protocols. Eluted MBP-AcpH was then concentrated to 10 mg/mL and subsequently FPLC-purified with 50 mM TrisCl and 250 mM TrisCl, pH 8.0, buffers to remove contaminating native E. coli MBP. MBP-AcpH was concentrated with a 10-kDa Amicon spin filter (Millipore) and stored in 40% glycerol at −80 °C after flash freezing aliquots in liquid nitrogen. 6×His-AcpH was lyzed in a similar manner, but it was purified with Ni-NTA resin (Novagen). Ni-NTA resin with bound protein was washed with 10 mM imidazole and eluted with 300 mM imidazole in lysis buffer. 6×His-AcpH was desalted to remove imidazole and flash frozen at −80 °C at 1 mg/mL without further modification. MBP-PaACP and Lux-ACP were expressed in E. coli BL-21 (DE3) in LB with 50 µg/mL kanamycin. GFP-ACP (6×His-tagged in pCA24N vector) was expressed in E. coli K-12 strain AG1 (ASKA library) cells in LB with 20 µg/mL chloramphenicol. Cells containing fusion ACPs were grown, induced and purified in an otherwise identical manner to 6×His-tagged AcpH. MBP and GFP fusion ACPs eluted with 300 mM imidazole were dialyzed into AcpH reaction buffer without Mg$^{2+}$ or Mn$^{2+}$ cofactors overnight. Lux-AcpH was buffer exchanged using a PD-10 desalting column (GE Healthcare) into AcpH reaction buffer, flash frozen and stored overnight at −80 °C. Lux-AcpH was thawed on ice, AcpH was added to 5 µM and the reaction was incubated at 37 °C for 4 h. Dialyzed ACP fusions next had appropriate amounts of 1 M MgCl$_2$ and MnCl$_2$ added to achieve 15 mM and 1 mM final concentrations, respectively. Free AcpH was added to free ACP and to MBP and GFP fusion ACPs at 5 µM final concentration, and the mixture was incubated overnight at 37 °C in a rotary wheel. Lux-AcpH was reacted for 4 h at 37 °C. AcpH reactions were centrifuged to remove any precipitate and purified by anion-exchange chromatography. Purity evaluation was conducted on MBP-PaACP and GFP-ACP (Supplementary Fig. 3) as well as on Lux-AcpH (Supplementary Fig. 4) with SDS-PAGE.

Preparation of coumarin-ACP standard. E. coli DK554 cells were grown, induced and prepared to generate predominantly apo-ACP. Isopropanol supernatant containing ACP was applied to DEAE resin and eluted with a sodium chloride gradient. ACP was then labeled using 6×His-Sfp and coumarin-CoA. Sfp was removed with Ni-NTA resin, and excess coumarin-CoA was removed with size-exclusion chromatography on G25 Sephadex resin. Preparation of E. coli DK554 lysate. 50 mL LB medium supplemented with 25 µM calcium d-pantothenate, 50 mM D-glucose and 50 µg/mL kanamycin used previously. Medium was inoculated with 1 mL of overnight DK554 starter culture and grown to OD = 0.4. IPTG was added at a concentration of 1 mM to the medium and was shaken for 5 h at 37 °C. Medium was centrifuged at 4,000 r.p.m. at 4 °C for 30 min to pellet cells. Cell pellets were resuspended in 25 mM TrisCl, pH 7.5; 250 mM NaCl;
0.1 mg/mL lysozyme; 10 μM pepstatin; and 10 μM leupeptin and passed twice through a French pressure device at 1,000 p.s.i. Lysate was centrifuged 45 min at 10,000 r.p.m. and 4 °C to remove insoluble debris.

Removal of coumarin-pantetheine from ACP in 5 mL lysate was achieved using 10 μM MBP-AcpH fusion in 600 mL AcpH reaction buffer (50 mM TrisCl, pH 8.0; 100 mM NaCl; 10% glycerol; 15 mM MgCl₂; and 1 mM MnCl₂) within a 3-kDa-MWCO dialysis bag at 37 °C overnight.

Fluorescence labeling of \textit{E. coli} DK554 lysate with modified coenzyme A. \textit{E. coli} DK554 cell lysate with total protein concentration of approximately 2 mg/mL was added to the volume of premade 10× PPTase reaction buffer (500 mM Na-HEPES and 100 mM MgCl₂, pH 7.6) that brought the total reaction concentration to 50 mM Na-HEPES, pH 7.6; 10 mM MgCl₂; 5 μM coumarin-CoA; and 2 μM Sfp. Samples were incubated at 37 °C for 1 h and then centrifuged to remove precipitate. Supernatant was passed over an equilibrated G50 Sephadex (GE Healthcare) desalting column, and lysate was dialyzed using 3.5-kDa MWCO membrane into 50 mM TrisCl, pH 8.0; 100 mM NaCl; and 10% glycerol to further remove unreacted CoA analog.

\textbf{AcpH treatment of coumarin-ACP in lysate.} Coumarin-labeled DK554 cell-lysate supernatant was added to a freshly prepared 10× AcpH reaction buffer to generate the following reaction concentrations: 50 mM TrisCl, pH 8; 150 mM NaCl; 15 mM MgCl₂; and 1 mM MnCl₂. MBP-AcpH was added to 2 μM. Reaction contents were placed in a 3.5-kd MWCO dialysis membrane and dialyzed against 50-fold volume of reaction buffer stirred overnight at 37 °C. No remaining coumarin-AcpH fluorescence was observed, and a substantial amount of MBP-AcpH appeared as precipitate afterward, as determined by SDS-PAGE analysis (not shown). Post-reaction contents were centrifuged 30 min at 4,000 r.p.m. at 6 °C. Supernatant was dialyzed back into 50 mM TrisCl, pH 7.5, and 250 mM NaCl in preparation for rhodamine labeling.

\textbf{Sfp and AcpH treatment of purified rhodamine-ACP.} The demonstrated activity of AcpH on rhodamine-ACP was performed with previously purified 6×His-tagged apo-ACP. First, 7 nmol of apo-ACP was treated with 5 μM native Sfp and 24 nmol of rhodamine-CoA and 24 mM native Sfp and 1 mM MnCl₂. MBP-AcpH was added to 2 μM. Reaction contents were placed in a 3.5-kd MWCO dialysis membrane and dialyzed against 50-fold volume of reaction buffer stirred overnight at 37 °C. Rhodamine-ACP was repurified with Ni-NTA resin to remove excess rhodamine-CoA and Sfp and dialyzed to remove imidazole. Dialyzed rhodamine-ACP was then incubated with and without 7 μM AcpH at 37 °C for 2 h, and the resulting crude reactions were run on SDS-PAGE and imaged to illustrate fluorescent-label removal with AcpH.

\textbf{AcpH treatment of \textsuperscript{15}NACP for NMR study.} An AcpH reaction was conducted to generate each apo-\textsuperscript{15}NACP sample before labeling. Following NMR acquisition of each sample, ACP was dialyzed into AcpH reaction buffer without cofactors (50 mM TrisCl, pH 8.0 and 100 mM NaCl). Glycerol was found to be unnecessary for desired AcpH activity and was omitted. Following dialysis, MgCl₂ and MnCl₂ were added to achieve a final concentration of 15 mM and 1 mM, respectively. Free 6×His AcpH was added to a concentration of 5–10 μM, and the mixture was incubated at 37 °C for 8 h. Reaction completion was determined by urea-PAGE analysis. The completed reactions were centrifuged 30 min at 4,000 r.p.m. at 6 °C to remove precipitate.

\textbf{Labeling of \textsuperscript{15}NACP using ‘one-pot’ Sfp methodology.} Apo-\textsuperscript{15}NACP was mixed with MBP-Coa, MBP-CoaD and MBP-CoaE, ATP disodium salt, native Sfp, and octanoyl-pantethenamide in a one-pot chemoenzymatic reaction to selectively generate octanoyl-\textsuperscript{15}NACP in vitro. Additional generation of \textsuperscript{13C}butanoyl-\textsuperscript{15}NACP and [8-\textsuperscript{13C}O]octanoyl-\textsuperscript{15}NACP analogs was conducted with the same methodology, except using regenerated apo-\textsuperscript{15}NACP with [13C]butanoyl-oxypantetheine and [8-13C]octanoyl-oxypantetheine. Ni-NTA resin was used to repurify the \textsuperscript{15}NACP after each labeling reaction. Monitoring of apo-, holo- and crypto-\textsuperscript{15}NACP was conducted with separation on conformationally sensitive urea-PAGE.

\textbf{ACP anion-exchange purification.} All preparative ACP samples were dialyzed into low-salt buffer. \textit{E. coli} ACP and MBP-PaACP ion-exchange running buffer was 25 mM- histidine, pH 6.0. GFP-ACP and Lux-ACP ion-exchange buffer was 25 mM bis-Tris, pH 6.0. Samples were then applied to a DEAE HiTrap (GE Healthcare) 1-mL or 5-mL column. Free ACP, MBP-ACP and GFP-ACP were loaded onto columns and washed with 10 mL of 25 mM buffer containing 25 mM NaCl and were eluted with 5 mL of 25 mM buffer and 500 mM NaCl. Lux-ACP was loaded onto a 5-mL DEAE HiTrap and washed with a step gradient of 0, 100, 200, 300 and 500 mM NaCl in 25 mM bis-Tris, pH 6.0. NMR \textit{E. coli} \textsuperscript{15}NACP samples were then dialyzed into 100 mM sodium phosphate and 1 mM DTT, pH 7.4, and then concentrated to 450 μL before NMR acquisition. \textit{E. coli} \textsuperscript{15}NACP NMR sample purity was evaluated by SDS-PAGE analysis. Following ion exchange, fusion ACPs were dialyzed or desalted, spin concentrated and stored at −80 °C before further use.

\textbf{Fusion ACP rhodamine-CoA labeling and label removal.} Purified apo-MBP-PaACP at 200 μM was labeled with 1 mM rhodamine-CoA and 13 μM native Sfp for 3 h at 37 °C. Purified apo-GFP-ACP at 150 μM was labeled with 1 mM rhodamine-CoA and 7 μM native Sfp for 3 h at 37 °C. Purified apo-Lux-ACP at 12 μM was labeled with 50 μM rhodamine-CoA and 3 μM native Sfp for 1 h at 37 °C. Fusion ACPs were then re-purified from excess rhodamine-CoA and native Sfp using Ni-NTA resin, buffer exchanged, and concentrated using 10-kDa MWCO 0.5 mL Amicon spin filters (Millipore). Label removal of MBP-ACP and GFP-ACP proceeded with 4 μL of the concentrated fusion ACPs with 10 μM AcpH in 10 μL AcpH reaction buffer for 3 h at 37 °C. For Lux-ACP, 6 μM crypto-Lux-ACP was reacted with 5 μM AcpH for 3 h at 37 °C. AcpH reaction samples were run immediately afterward on urea-PAGE (MBP-ACP and GFP-ACP) or SDS-PAGE (Lux-ACP) with no further purification.

\textbf{Sfp and AcpH treatment of \textit{E. coli} ACP and fusion ACPs for efficiency analysis.} Labeling of butanoyl-, octanoyl- and coumarin-ACP proceeded via Sfp and CoA-A/D/E one-pot methodology. Labeling of free ACP and fusion ACPs with rhodamine proceeded via Sfp and rhodamine-CoA. Free \textit{E. coli} ACP reactions were conducted overnight at 37 °C. MBP-PaACP labeling proceeded via native Sfp; GFP-ACP and luciferase-ACP reactions using Sfp conjugate proceeded for 6 h at 37 °C. MBP-PaACP was repurified.
using Ni-NTA and desalted with PD-10 desalting column to remove imidazole. GFP-ACP and luciferase-ACP were separated from solids with a fritted spin column and desalted to remove excess CoA analog. Nonfluorescent *E. coli* ACPs were quantified using UV spectrometry. Label removal proceeded in AcpH reaction buffer with 10% glycerol via reaction with an AcpH conjugate for *E. coli* ACP at 37 °C overnight and at room temperature overnight for MBP-PaACP and GFP-ACP. Because of precipitation induced by extended incubation, luciferase ACP was reacted with AcpH at 37 °C for 2 h. Reaction completion was monitored by gel shifts and/or fluorescence depletion in 20% urea-PAGE for free ACPs and 10% SDS-PAGE for the fusion ACPs. Final apo-ACP samples were then desalted into AcpH reaction buffer lacking Mg²⁺/Mn²⁺ and subsequently quantified (Table 1).

**GFP-ACP: rhodamine-CoA and Sfp labeling monitoring by FRET.** Apo-GFP-ACP and rhodamine-CoA were diluted to 100 μM and 200 μM, respectively, in 10 mM TrisCl, pH 7.5. Of this 10× substrate mix, 5 μL was added to 6 wells of a Costar 3694 96-well plate (Corning) in triplicate. To adjust final concentrations, 10 μL of milliQ water diluent was added. Next, 35 μL of 1.43 μM Sfp in 71.5 mM HEPES, pH 7.6; 14.3 mM MgCl₂; and 1.43 mg/mL BSA (stabilizer) was added to initiate the reaction, with reaction buffer lacking Sfp added to the control. Final 50-μL enzyme reactions contained 10 μM apo-GFP-ACP; 20 μM rhodamine-CoA; 1 μM Sfp; 50 mM HEPES, pH 7.6; and 10 mM MgCl₂. The 96-well plate was centrifuged 2 min at 1,000 r.p.m., and fluorescence was monitored at 405 nm excitation and 595 nm emission for 30 min in a PerkinElmer HTS 7000 Plus plate reader at room temperature.

**Pantetheine probe synthesis.** All CoA-related probes are depicted in Supplementary Figure 18. Pantetheine probe synthetic methods and chemical spectra are also contained within the Supplementary Note and Supplementary Figures 19–42.

21. Kitagawa, M. *et al.* **DNA Res.** **12,** 291–299 (2005).
22. Lambalot, R.H. & Walsh, C.T. **J. Biol. Chem.** **270,** 24658–24661 (1995).
23. Foley, T.L. & Burkart, M.D. **Anal. Biochem.** **394,** 39–47 (2009).
24. Foley, T.L. *et al.* **Org. Biomol. Chem.** **8,** 4601–4606 (2010).
25. Worthington, A.S. & Burkart, M.D. **Org. Biomol. Chem.** **4,** 44–46 (2006).