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Resin Supported Acyl Carrier Protein Labeling Strategies

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The post-translational modifying enzymes phophopantetheinyl transferase and acyl carrier protein hydrolase have shown utility in the functional modification of acyl carrier proteins. Here we develop these tools as immobilized biocatalysts on agarose support. New utility is imparted through these methods, enabling rapid and label-independent protein purification. Immobilization of acyl carrier protein is also demonstrated for rapid activity assays of these 4'-phosophopantetheine modifying enzymes, displaying a particular advantage in the case of phosphopantetheine removal, where few orthogonal techniques have been demonstrated. These tools further enrich the suite of functional utility of 4'-phosophopantetheine chemistry, with applications to protein functionalization, materials, and natural product biosynthetic studies.

INTRODUCTION

The acyl carrier protein (ACP) plays a central role in the biosynthesis of fatty acid and polyketide synthase natural products as a tethered chaperone for growing substrates.[1] For this reason, ACP is found to play a pivotal role in many *in vitro* biochemical evaluations, and new findings about its molecular activity continue to be discovered.[2] In 2004, we introduced the post-translational modification of ACP with a phosphopantetheinyltransferase (PPTase) and functional coenzyme A (CoA) analogues in order to attach 4'-phosphopantetheine (PPant) with unique properties, and these tools have been leveraged for enzymology studies and the functionalization of proteins.[3] In addition, we recently introduced the use of an ACP hydrolase (AcpH) to cleave PPant and PPant analogues from ACP as a reversible labeling tool, thus providing a full suite of ACP modifying methodologies.[4] Here we further coordinate this dual enzyme utility as immobilized biocatalytic tools, thereby streamlining their application for protein labeling, unlabeling, and isolation.

Site-specific enzymatic labeling of stand-alone ACPs and ACP-fusions has found utility in many applications: elucidation of truncated ACP substrates,[5] mammalian cell labeling[6] endogenous ACP discovery,[7] enzymology,[8] and structural studies.[3] These modifications rely on the availability of ACP in the *apo-* form, a state often limited by the conversion of heterologously expressed *apo*-ACP to *holo*-ACP by endogenous PPTases.[9]

Prior to this work, separation of *apo-* and *holo-* forms of ACP required size exclusion chromatography or thiol-sepharose resin. The latter technique is only viable for proteins lacking surface cysteine residues, such as the ACP from *Escherichia coli* fatty acid biosynthesis, AcpP (accession no. AAC74178). Here, the thiol resin forms a disulfide bond with the terminal sulfhydryl of

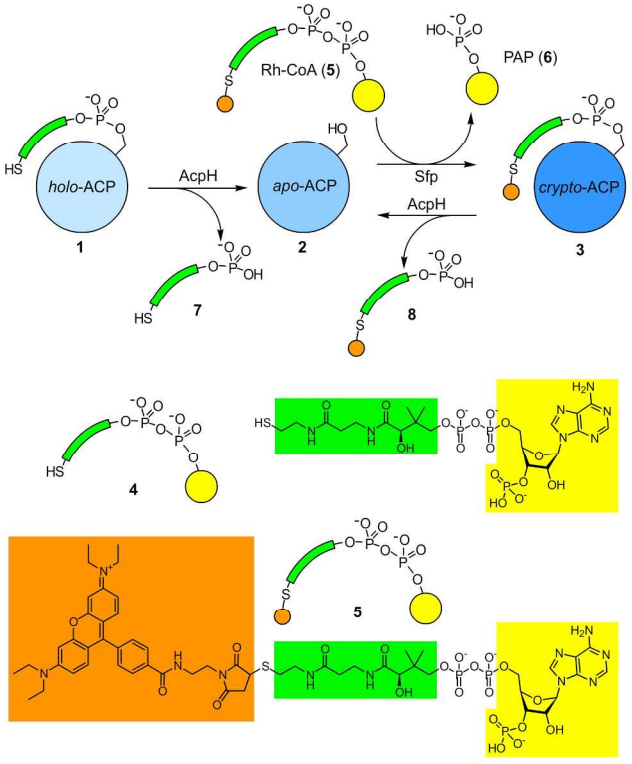

Fig. 1 Acyl carrier protein hydrolase and phosphopantetheinyl transferase reactions. Endogenous *holo*-ACP can be converted to *apo*-ACP by AcpH. Sfp utilizes *apo*-ACP and coenzyme A or coenzyme A analogues such as rhodamine-CoA **5** as substrates to catalyze the transfer 4'-phosphopantetheine onto the active serine residue of ACP generating *crypto*-ACP.

PPant, leaving the unmodified *apo-* form unbound. Although effective, large preparations require multiple iterations of purification to completely separate *holo*-ACP from the

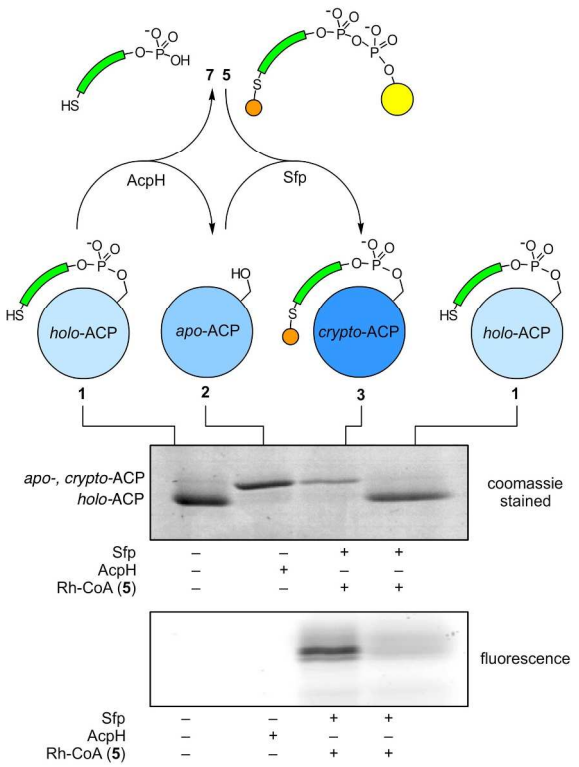

Fig. 2 Demonstration of on-resin activity of AcpH and Sfp. *Holo*-ACP **1** is first converted to the *apo*- form **2** by treatment with resin-bound AcpH., The same sample is subsequently labeled by treatment with resin-bound Sfp and rhodamine-CoA **5.** Lane 4 serves as a negative control; it is **1** treated with resin-bound Sfp and **5** without prior AcpH treatment. In these samples, no Sfp or AcpH is observed. Minor fluorescent background is observed in lane 4 due to the cross-reactivity of the maleimide in **5** with the free sulfhydryl on *holo*-ACP. These transformations are visualized by urea-PAGE which separates ACP forms based on their conformational shape.

apo/holo-ACP mixture. When the *apo*-ACP is desired, the *holo*-form of the purified protein goes unused as an expensive side product, a particularly wasteful procedure when isotopes are incorporated into the protein for biophysical studies such as NMR.[3] For these reasons, the conversion of modified ACP to *apo*- form by AcpH is an attractive purification strategy to provide homogenous products. Here, AcpH cleaves the PPant appendage from both *holo*- and *crypto*- (or functionally labeled) ACP, resulting in uniformly *apo*- protein. (Fig. 1)

First identified for activity by Vagelos in 1967, the AcpH gene sequence remained mis-annotated for over 30 years,[10] and the *E. coli* homolog proved mostly insoluble when overexpressed.[11] Fortunately, a homolog of AcpH identified from *Pseudomonas aeruginosa* can be solubly expressed as an active recombinant protein.[12] We further fused this recombinant protein with various MBP and His-Tagged fusions and demonstrated activity upon a variety of modified PPant analogues, including fluorescent and natural product mimics.[4] We focus on the C-terminal His-tagged construct as the most versatile form.

In order to purify a desired product from a mixture of proteins, discrete purification proceeds either by the use of orthogonal fusion tags (His_6, MBP, or Strep.) or by taking advantage of physical properties of the protein of interest such as molecular weight or isoelectric point.[13] These traditional methods become subjected to potential contamination in the form of nonspecific binding interactions and overlapping physical properties. Overall

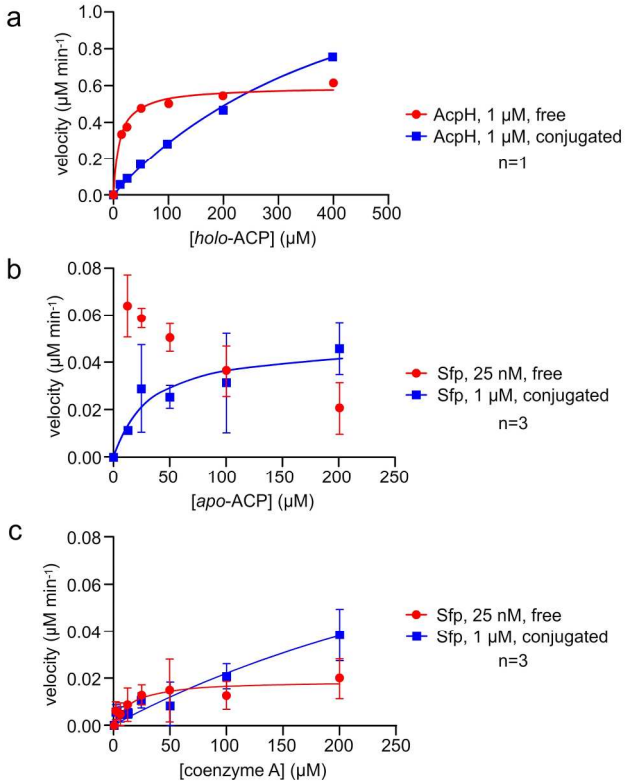

Fig. 3 Basic Michaelis-Menten kinetics of free and conjugate enzymes were evaluated for AcpH/*holo*-ACP (a), Sfp/*apo*-ACP (b), and Sfp/CoA (c).

yield invariably suffers from subsequent purifications to eliminate contaminants at every step.

His-Tag purification is an attractive option due to its versatility and high-yielding purification. However, most ACPs are also prepared as His-tagged fusions, as are the most useful recombinant PPTases. Therefore one-step, orthogonal purification away from His-PPTase and His-AcpH is not easily achievable. Further, AcpH has been observed to co-purify with endogenous fatty acid ACP,[4] a phenomenon that will inevitably contaminate even orthogonally tagged constructs.

RESULTS AND DISCUSSION

Enzyme immobilization circumvents potentially detrimental purification strategies and allows for isolation of pure substrate samples after removal of catalytic enzymes by simple centrifugation or filtration. Additionally, enzyme immobilization is often used in industrial processes as a way to recycle enzymes.[14]

Other studies have shown that polymeric attachment can promote protein solubilization and stability[15] although this result can vary based on the protein identity and the conjugation method.

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Table 1 Kinetic parameters for AcpH and Sfp enzymes

enzyme	substrate	state	[substrate] (μM)	V_{max} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
AcpH	*holo*-ACP	free	1.0	0.59±0.02	12±2	0.59±0.02	0.049
		conjugate	1.0	1.7±0.2[a]	490±70[a]	1.7±0.2 [a]	0.0035
Sfp	*apo*-ACP	free	0.025	0.45±0.01[b]	NC[c]	2.55±0.53	NC[c]
		conjugate	1.0	0.048±0.010	31±20	0.048±0.010	0.0015
	CoA	free	0.025	0.019±0.003	16±9	0.79±0.34	0.0012
		conjugate	1.0	0.14±0.12[a]	510±570[a]	0.14±0.12[a]	0.0003

[a] V_{max} and K_m values for enzyme conjugates are derived from processed data derived from concentration ranges below the calculated K_m.

[b] V_{max} and k_{cat} for free Sfp with varied [*apo*-ACP] are derived from the lowest ACP concentration (12.5 μM), due to decreasing activity with higher ACP concentrations.

[c] NC denotes that this value could not be calculated correctly, due to decreasing activity with higher ACP concentrations.

For the purpose of studies that require iterative ACP labeling, it would also be attractive to quickly and efficiently cycle on-and-off PPant substrates while maintaining the modifying enzymes. Therefore, we chose to prepare immobilized versions of the AcpH and PPTase enzymes.

AcpH and Sfp conjugates are made by incubating the Ni-NTA purified protein, buffered in 50 mM MOPS, pH 7.5, with Affi-gel 10 (Bio-Rad, Hercules, CA), an agarose resin that is derivatized with NHS-activated esters. This resin conjugates proteins by displacing the NHS-ester with a free amine, the source of which is a surface lysine residue or at the *N*-terminus. After coupling, the resins are capped with 100 mM ethanolamine, pH 8.0 and extensively washed in 50 mM Tris, pH 8.0 to remove any unbound protein. The washing step ensures that no exogenous protein is introduced during modification. We have observed that this coupling procedure is very efficient and conjugates virtually all of the total protein in solution, as monitored by Bradford or by SDS-PAGE analysis. (Fig. S1A)

The sustained activity of AcpH and Sfp conjugates can be observed by gel electrophoresis analysis. Change in the identity of the ACP (whether *apo*-, *holo*-, or *crypto*-) is observed by conformationally sensitive urea-PAGE.[9] When using the AcpH conjugate, we observed the conversion of *holo*-ACP to *apo*-ACP without any contaminating proteins. Following filtration, *apo*-ACP can be subsequently labeled with a CoA analogue. In this study we labeled our newly converted *apo*-ACP with a fluorescent analogue, rhodamine-CoA **5**.[16] Here, Sfp-agarose conjugate appends rhodamine-PPant, and the conversion to the fluorescent *crypto*- form is observed by SDS-PAGE without any contaminating Sfp (Fig. 2).

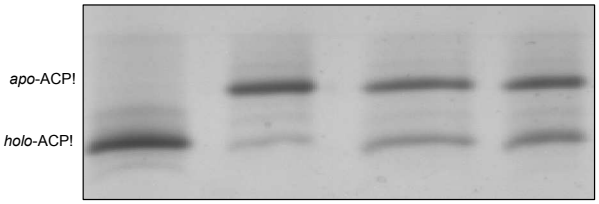
We further investigated AcpH and Sfp conjugates by enzyme kinetics. Although enzyme immobilization may impart limitations onto the catalytic activity in comparison to their soluble counterparts, it has been suggested that measuring Michaelis-Menten kinetics may still be used as an evaluation method.[17] Using an HPLC-based assay to monitor the conversion of *apo*- to *holo*-ACP by PPTase and the reverse process by AcpH, we found that both enzymes retain activity as conjugates, but with altered kinetic parameters. (Table 1) As compared to the free, unconjugated AcpH k_{cat}/K_m of 0.049, the conjugated form has a k_{cat}/K_m of 0.004. This results in part from a very high-calculated K_m of 490 μM. One likely cause for such a high K_m is the limited ability for immobilized proteins to diffuse as freely as proteins in the solution phase. Interestingly, the conjugated AcpH possesses a higher maximum k_{cat} of 1.7 ± 0.2 min^{-1} compared to 0.59 ± 0.02 min^{-1} for the free AcpH. (Fig. 3a).

Overall, the benefit of AcpH conjugation to effectively turnover high concentrations of *holo*-ACP is balanced by slightly slower reaction rates at low substrate concentrations. Conjugation of Sfp introduces larger kinetic penalties than AcpH, possibly owing to a bisubstrate reaction instead of the monosubstrate reaction of AcpH. The large discrepancy in activity between the free and conjugate Sfp necessitated a significant difference in concentrations for the kinetic analysis, with 25 nM free Sfp versus 1 μM conjugated Sfp. Additionally, Sfp activity was slowed from optimal rates by using a higher than optimal pH of 7.6 to approximate "one-pot" custom pantetheine labeling conditions. The maximum k_{cat} values of free Sfp are 2.55 ± 0.53 min^{-1} and 0.79 ± 0.34 min^{-1} from *apo*-ACP (Fig. 3b, S2a) and CoA dilution (Fig. 3d, S2b) series, respectively. These values drop to 0.048 ± 0.010 min^{-1} and 0.14 ± 0.12 min^{-1} upon treatment with the immobilized enzyme. The discrepancy in k_{cat} values from the CoA and *apo*-ACP dilutions arises from the observed phenomenon whereby Sfp activity decreases with *apo*-ACP concentrations higher than 12.5 μM. This substrate inhibition phenomenon has been observed previously for *E. coli* ACP[18] and prevented the calculation of K_m within the experimental limitations of the HPLC assay. Therefore, we could not compare

k_{cat}/K_m values between the free and conjugate Sfp. Regardless, the Sfp conjugate kinetic data indicates a similar qualitative shift towards higher V_{max} and K_m seen in AcpH conjugates. Furthermore, it has been observed that the AcpH enzyme conjugates retain activity when held at 4 ºC for greater than 4 weeks, while Sfp conjugates degrade rapidly and are almost completely inactive 1 week post-conjugation. We hypothesize that the instability of Sfp is due to its inherent flexibility as a soluble protein and the previously observed property of insolubility with most fusion proteins.[19]

Our enzyme conjugates lend themselves to enzyme recycling for repeated *apo*-ACP purification. The utility of using an ezyme conugate is found when the modifying enzyme is particularly hard to obtain or for overall convenience. We tested the ability of AcpH to generate *apo*-ACP after an abbreviated 2 h incubation. The effiicacy of the enzyme conjugate was shown to decrease washing and repeated reactions, but sustained moderate levels of activity for three cycles. (Fig.4)

In addition to the tailoring enzymes that modify the form of ACP, ACP itself can be used as an immobilized protein for the evaluation of tailoring enzyme activity. The preparation of resin-bound ACP was first used as an affinity matrix for ACP partners such as the *holo*-ACP synthase (AcpS.)[20] The preparation of modified and immobilized ACP has been used more recently for the identification of selective partners from *E. coli*, where Sfp was demonstrated to modify resin-bound ACP.[21] Here we demonstrate that immobilized ACP conjugates are also capable of modification by AcpH.



	No AcpH Treatment!	1st Cycle!	2nd Cycle!	3rd Cycle!
apo-ACP!	0%!	91%!	68%!	60%!
holo-ACP!	100%!	9%!	32%!	40%!

Fig. 4 Recycling of immobilized AcpH. Multiple samples of *holo*-ACP can be converted to *apo*-ACP using the same AcpH conjugate. After generation of *apo*-ACP by 2 h incubation with AcpH conjugate, the beads are washed and introduced into a separate sample of *holo*-ACP.

Immobilized *apo*-ACP was prepared as previously reported, and Sfp catalyzed labeling was performed using rhodamine-CoA analogue **5**. After washing, fluorescence indicative of PPTase activity was observed on the beads by placing a small sample on a fluorescent microscope. After fluorescence confirmation, the signal was subsequently removed by treatment with soluble AcpH. This process could be iterated, such that the resin could be washed again and the cycle repeated (Fig. 5). In the authors' experience, this technique was extremely rapid, particularly in regard to the removal of the fluorescent probe. Upon addition of AcpH, activity was qualitatively confirmed almost immediately by observation of the diffusion of the fluorescent probe off of the resin and into solution.

Conclusions

The ACP lends itself very well to bioconjugate chemistry. It is a unique protein that may be post-translationally modified into many forms. Rapid transformation and purification to functional forms offers the rapid conversion and use these of species. As this study demonstrates, immobilization of tailoring enzymes can greatly streamline this process. In addition, the immobilization of ACP enables rapid evaluation substrates and modifying enzymes for ACP functionalization. As ACP biochemistry continues to be explored in the paradigm of diverse substrate loading, these techniques will aid researchers in quickly preparing and recycling samples.

Experimental

Protein Immobilization.

Immobilization of all proteins was carried out according to the manufacturer's instruction. Briefly, proteins would be either dialyzed or desalted into 50 mM MOPS buffer, pH 7.5. After washing the resin with ice cold H_2O, (3 × 1 mL) the resin would be transferred to a 1.7 mL eppendorf tube and an equal volume amount of protein would be added. The protein was allowed to immobilize overnight at 4 ºC with agitation. The reaction was then capped with 1 M ethanolamine, pH 8.0 by the addition of 0.1 mL per volume of resin used. After 1 h incubation at 4 ºC, the resin was transferred to a 15 mL falcon tube and washed with 50 mM Tris, pH 8.0 (3 × 10 mL) with inversion for 1 min at RT, centrifugation (800 × g, 2 min), and removal of the supernatant. The resulting resin was stored at 4 ºC.

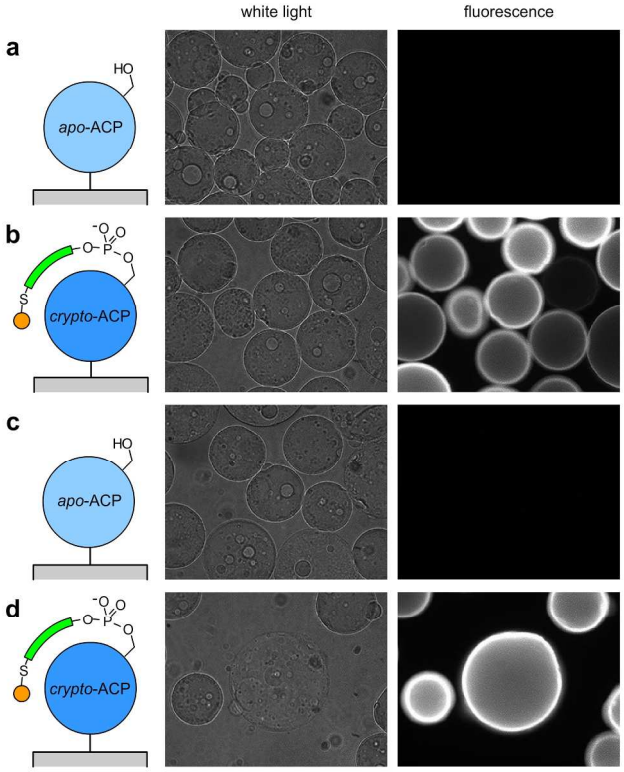


Fig. 5 Iterative labeling of immobilized ACP. Immobilizing ACP allows for rapid qualitative activity assays of PPTase and AcpH enzymes. After immobilized *apo*-ACP is labeled with **5** there is an observable fluorescent signal. This fluorescence can be cycled on and off by alternating treatment with AcpH and PPTase.

ACP Expression and Purification.

Apo- and *holo*- ACP standards were prepared by overexpression of *E. coli* AcpP in pET22a vector (EMD Millipore) in BL-21 (DE3). Cells were grown in LB with 100 μg/mL ampicillin at 37 °C until they reached an optical density of 0.6. IPTG was added to 1 mM, and the cells were incubated with shaking at 16 °C for 12 h. A mixture of *apo*-/*holo*- ACP was obtained through Ni-NTA chromatography, and the resultant protein was split into a portion treated with AcpH to generate the *apo*- and a portion treated with Sfp and excess CoA to generate *holo*-ACP at 37 °C for 12 h. Both *apo*- and *holo*-ACP were then further purified from AcpH/Sfp with size exclusion chromatography. Spin concentration of ACP with 3 kDa centrifugal filters (EMD Millipore) resulted in a stock of *apo*-ACP at 3 mM and *holo*-ACP at 1.5 mM. Concentrations were determined using UV spectroscopy at 280 nM with the extinction coefficient of 1490 $M^{-1}cm^{-1}$ calculated from the ExPASy (http://www.expasy.org/) ProtParam tool.

AcpH Expression and Purification.

AcpH plasmid construct, growth, and purification procedures were performed as previously described[4] with the exception of lysis buffer. AcpH lysis buffer was 50 mM MOPS, pH 7.6, 250 mM NaCl, 10% glycerol, 0.1 mM DTT.

Labeling Free ACP with Sfp Resin.

Apo-ACP (100 μg) is mixed with 50 μL Sfp beads (~5 μg/μL), 12.5 mM $MgCl_2$, 50 mM Tris, pH 8.0 and 20 μM rhodamine-mCoA. The reaction is allowed to proceed at 37 ºC for at least 4 h with agitation. The Sfp resin is removed by microfuge centrifugation.

Unlabeling Free ACP with AcpH Resin.

Crypto-ACP (50 μg) is mixed with 50 μL AcpH beads (~1 μg/μL), 1mM $MnCl_2$, 12.5 mM $MgCl_2$, 50 mM Tris, pH 8.0. The reaction is allowed to proceed at 37 ºC for at least 1 h with agitation. The AcpH resin is removed by microfuge centrifugation. Reaction is monitored by urea-PAGE analysis.[9]

Labeling ACP Resin with Free Sfp.

Immobilized *apo*-ACP (100 μL Affigel-15 bed volume, 100 μg immobilized) is mixed with 2 μM Sfp, 20 μM rhodamine-CoA, 12.5 mM $MgCl_2$, 50 mM Tris, pH 7.5 in a total reaction volume of 250 μL. The reaction is allowed to dwell at 37 ºC for at least 1 h to generate *crypto*-ACP. The beads are washed with 1 M NaCl (3 × 500 μL) and 50 mM Tris, pH 8.0 (3 × 500μL) to remove excess rhodamine-CoA and Sfp.

Unlabeling ACP Resin with Free AcpH.

Immobilized *crypto*-ACP (50 μg) is mixed with 2 μM AcpH, 1 mM $MnCl_2$, 12.5 mM $MgCl_2$, 50 mM Tris, pH 8.0 in a total reaction volume of 100 μL. The reaction is allowed to proceed at 37 ºC for at least 1 h although pink diffusion due to the removal of rhodamine-CoA is observed after only 5 min. The beads are washed with 1 M NaCl (3 × 500 μL) and 50 mM Tris, pH 8.0 (3 × 500 μL) to remove excess rhodamine-PPant **8** and AcpH.

AcpH Kinetics Sample Preparation.

Holo-ACP was diluted into 50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 10% glycerol, 30 mM $MgCl_2$ and 2 mM $MnCl_2$ buffer to a concentration of 800 μM. Serial dilution of *holo*-ACP resulted in a final concentration range of 800-25 μM. AcpH was diluted from MOPS lysis buffer into 50 mM Tris-Cl, pH 8.0, 250 mM NaCl, 10% glycerol and added to an equal volume of the *holo*-ACP serial dilution to initiate the reaction. Reaction tubes were transferred to a pre-warmed rack at 37 °C and agitated for the duration of the experiment. Time points were collected at 10, 20, 30, 45, 60, 90 min by addition of reaction contents to 100 mM EDTA, pH 8.0. In order to ensure consistent removal of AcpH-conjugate resin, cut-off pipette tips were used to resuspend reaction contents and homogenously remove enzyme with the reaction contents. All samples were frozen at -80 °C until evaluated by HPLC.

Sfp Kinetics Sample Preparation.

Serial dilutions of *apo*-ACP and CoA were prepared separately in 50 mM MOPS, pH 7.6, 250 mM NaCl, 10% glycerol, resulting in a final concentration range of 800-50 μM for *apo*-ACP and 800-6.5 μM. The 800 μM of coenzyme A was added to *apo*-ACP serial dilution, and 800 μM *apo*-ACP was added to CoA serial dilution for "saturating" concentrations, resulting in a concentration range of 400-25 μM for each serial dilution prior to enzyme addition. Sfp was diluted in MOPS buffer to free Sfp concentration of 50 nM, a conjugate Sfp concentration of 2 μM, and 20 mM $MgCl_2$. The Sfp enzyme mixtures were then added in an equal volume to the combined *apo*-ACP/CoA substrate mix, diluting to final concentrations of 25 nM for free Sfp, 1 μM for conjugate Sfp, ranges of 200-12.5 μM *apo*-ACP (with 200 μM CoA) and 200-1.6 μM CoA (with 200 μM *apo*-ACP). Time points were collected for free Sfp at 30 min in triplicate, and at 30/60/120/240/360 min for conjugate Sfp in singlet.

HPLC Methods.

Thawed kinetics samples were mixed briefly by flicking, and centrifuged (13,000 rpm, 10 min) at RT prior to transferring contents into HPLC vials. 20 μL of each reaction time point was injected on an Agilent 1100 series HPLC with OD5 # 9575, 25 cm x 4.6 mm ID column (Burdick & Jackson) using an acetonitrile/water gradient. Both water and acetonitrile contained 0.05% TFA. Method gradient for each injection: 0-5 min with 10% acetonitrile, 5-30 min with 10-100% acetonitrile, 30-35 min with 100% acetonitrile, 35-37 min with 100-10% acetonitrile, 37-40 min with 10% acetonitrile. HPLC-grade solvents (J.T. Baker) were used exclusively. *Apo*- and *holo*-ACP protein standards were used to validate the retention times identified using 210 nm UV light at approximately 21 and 19 min, respectively. A small amount of *holo*-ACP (under 1%) was present in *apo*-ACP stock used for Sfp kinetics, and was background subtracted from calculations. Peak integration was performed for all samples, and substrate turnover was calculated from the ratio of *apo*- to *holo*-ACP present in the HPLC trace and the known concentration of total ACP in reaction samples. Calculated rates for AcpH versus substrate concentration were obtained through Excel data analysis and graphed in Prism GraphPad using the "Michaelis-Menten" function for enzyme kinetics, with a zero data point added for substrate concentration of 0 $\mu M*min^{-1}$ and 0 μM substrate for both AcpH and Sfp graphs.

Microscopy.

Fluorescent microscopy was conducted on an Eclipse TE300 (Nikon) using irradiation from mercury vapor lamp. Blue fluorescence was collected using a dichroic filter set with excitation filtered at 377 nm BP 50 and emission filtered at 477 BP 60 (FF409, Semrock). Red fluorescence was collected using a dichroic filter set with excitation filtered at 562 nm BP 40 and emission filter at 624 BP 40 (FF506, Semrock).

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Notes and references

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† Electronic Supplementary Information (ESI) available: Copies of original images of gels from Figure 2. See DOI: 10.1039/b000000x/

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