Probing the Phosphopantetheine Arm Conformations of Acyl Carrier Proteins using Vibrational Spectroscopy

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Supporting Information

MATERIALS AND METHODS

General

All chemical reagents were purchased from Sigma Aldrich unless otherwise noted. Ni-NTA slurry and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Gold Biotechnology. Plasmid prep kits were purchased from Qiagen. SDS was bought from Bio-Rad and the LB-Miller powder came from IBI Scientific. Proteins were concentrated in an Allegra X-14R centrifuge (Beckman Coulter). Cells were harvested in an Avanti J-E centrifuge (Beckham Coulter) during the protein expression process. Two types of 3kDa-cutoff Centricons were used to concentrate proteins: Amicon Ultra centrifugal units (Millipore) and Vivaspin columns (2 mL capacity, Sartorius Stedim Biotech). The actinorhodin ACP plasmid (MC002067) was provided by the Chang Lab at Berkeley and the DEBS ACP2 plasmid (pNW7) was provided by the Khosla Lab at Stanford.

Preparation and purification of ACPs

The following plasmids were transformed into chemically competent BL21 and BAP1 *E. coli* cell lines in order to express apo and holo ACPs, respectively: pNW7 (DEBS ACP2, kanamycin resistance), MC002067 (ACT ACP, kanamycin resistance), and GmCD00338278 (GmACP3, carbenicillin resistance). Seed cultures grown overnight at 37 °C in 10 mL of LB media (100 μg carbenicillin/mL, 50 μg kanamycin/mL) were added to 1-L LB production cultures (100 mg carbenicillin/L, 50 mg kanamycin/L). Production cultures were grown at 37 °C until OD_{600} = 0.5-0.7 before being induced with 250 μL of 1 M IPTG. Induced cultures were incubated with shaking at 18 °C overnight. The cells were then harvested by centrifugation (4500*g*, 15 min), resuspended in lysis buffer (50 mM phosphate, pH 7.6, 300 mM NaCl, 10 mM imidazole), and sonicated using an XL-2000 Microson sonicator (cells on ice, 8 x 30 sec pulses with 30 sec rest in between, power level = 12). Cell debris was removed by centrifugation (17000*g*, 90 min). Nickel-NTA agarose slurry (Ni-NTA resin in 20% EtOH) was added

to the supernatant (3 mL slurry /L culture) and mixed for one hour at 4 °C. The resin was loaded into a fritted column and the flow through was collected. The resin was washed twice with 20 mL of wash buffer (50 mM phosphate, pH 7.6, 300 mM NaCl, and 30 mM imidazole) and then the desired N-terminal His-tagged proteins were eluted with 6 mL of elution buffer (50 mM phosphate, pH 7.6, 100 mM NaCl, and 150 mM imidazole). The protein-containing eluate was initially concentrated using a 3-kDa cutoff Millipore Centricon. The elution buffer was exchanged with storage buffer (50 mM phosphate, pH 7.6) and reconcentrated in the smaller Vivaspin Centricon. Proteins were then aliquoted and flash-frozen in liquid nitrogen and stored at -80 °C

Cyanylation of ACPs

Holo ACP aliquots were combined and diluted with 50 mM phosphate buffer (pH 7.0) to about 0.2 mM protein concentration as determined using UV spectroscopy. The protein solution was treated with 8 equivalents of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) dissolved in 200 mM phosphate buffer (pH 7.0) for 90 minutes to form a mixed disulfide at the terminal thiol of the 4'-Ppant arm. Addition of DTNB solution resulted in the protein solution turning a light yellow color due to the release of the TNB byproduct that absorbs at 412 nm. The DTNB-ACP adduct sample was then treated with 55 molar equivalents of NaCN dissolved in 50 mM phosphate buffer (pH 7.0) for 45 minutes. Addition of the NaCN solution resulted in the solution turning a more intense yellow color due to further release of TNB and generation of cyanylated DTNB species. The cyanylated ACP was isolated using a Sephadex PD-10 desalting column (GE Healthcare) equilibrated in the same 50 mM phosphate buffer (pH 7.0). Cyanylated ACP was concentrated to 0.5-1.0 mM with a 3-kDa cutoff Vivaspin centrifugal concentrator (Sartorius).

Far-UV CD

CD spectra were collected from 180 to 260 nm (one point per nm) using an Aviv model 410 spectropolarimeter. Cyanylated and unmodified ACP samples were diluted to 4-5 μM concentration using a 5 mM phosphate buffer (pH 7.6). Samples were analyzed in a 1 mm quartz demountable cell (Starna Cells).

Infrared Spectroscopy

Cyanylated ACP aliquots were combined and concentrated to 20-30 μL and 0.5-1 mM using a 3-kDa cutoff Vivaspin centrifugal concentrator. About 8-10 μL of the concentrated sample was placed between the windows of a Harrick 13 mm diameter demountable liquid cell using polished CaF_2 windows and a path length of 50 μM set using a Teflon spacer. All spectra were collected at 2 cm^{-1} resolution (512 scans) between 1900-2700 cm^{-1} using a Bruker Optics Vertex 70 FTIR spectrometer with a photovoltaic MCT detector. A spectrum of 50 mM phosphate buffer solution was used as the background, and further baseline correction was accomplished by fitting the baseline (outside the region from 2145 to 2180 cm^{-1}) to an 8^{th} degree polynomial and subtracting the fit from the raw absorbance spectrum.

RESULTS

Far-UV CD spectra

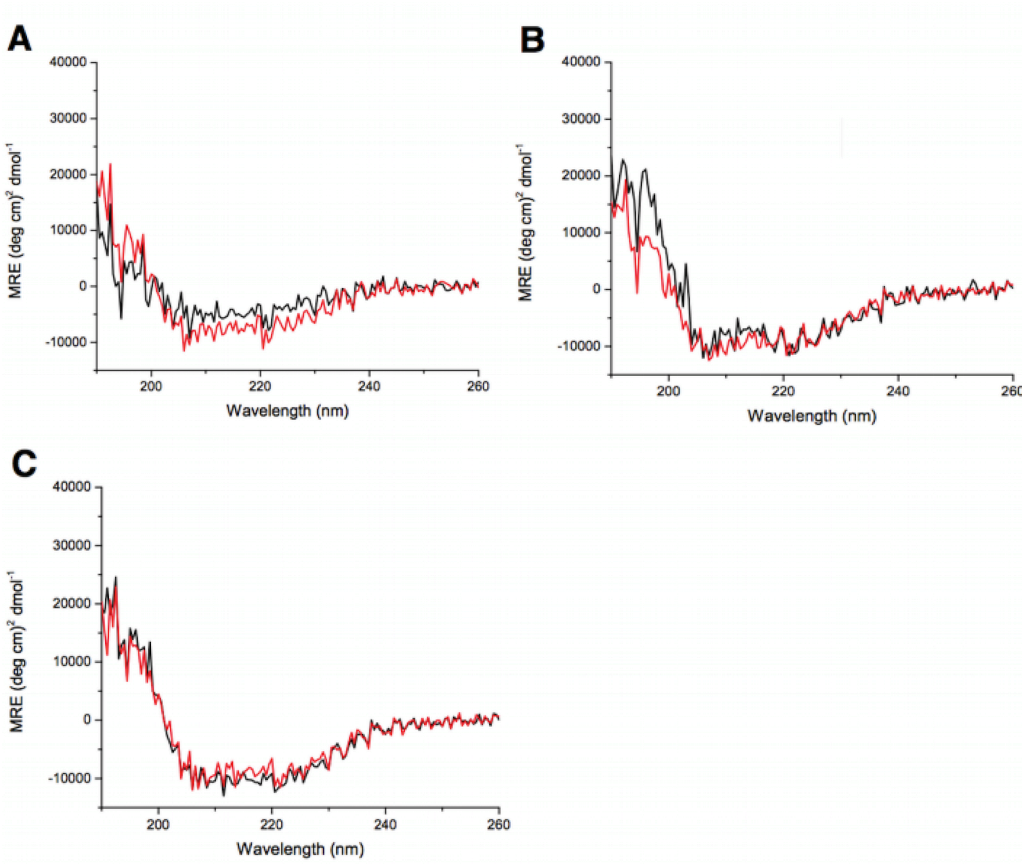

Figure S1. Far-UV CD data of all unmodified (black) and cyanylated (red) ACP proteins in phosphate buffer. A) GmACP; B) ACT ACP; C) DEBS ACP2. In all cases, the cyanylated protein's spectrum is nearly identical to that of the unmodified protein within the signal:noise of the instrument.

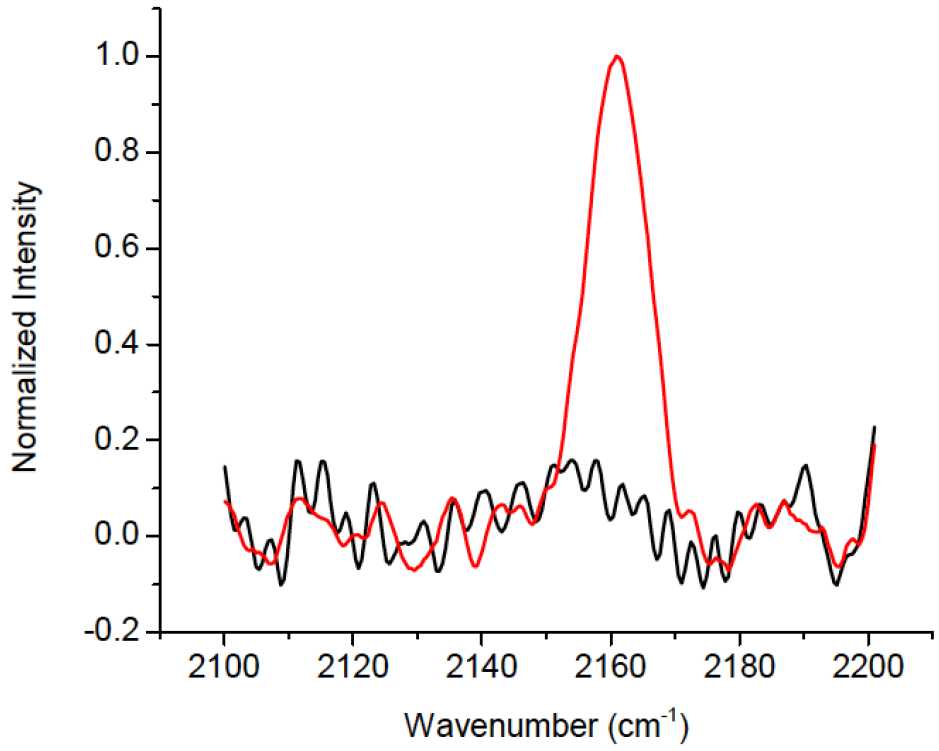

Figure S2. Comparison of the IR spectra of the cyanylated apo ACT ACP (black) to the cyanylated holo ACT ACP (red). The lack of a peak in the CN band region in the apo ACT ACP spectra indicates that Cys17 is not cyanylated during the reaction with DTNB and NaCN.

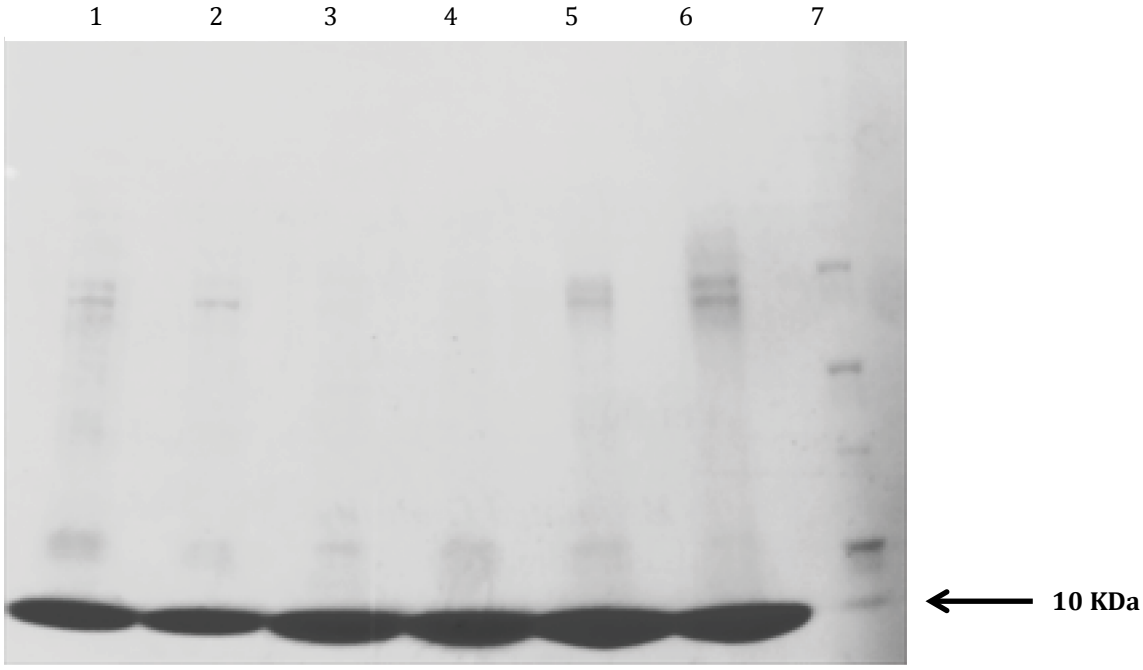

Figure S3. SDS-PAGE gels of purified ACPs (1, holo DEBS ACP2; 2, apo DEBS ACP2; 3, holo ACTACP; 4, apo ACT ACP; 5, holo GmACP3; 6, apo GmACP3; 7, Precision Plus Protein Standards).