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Metabolic Perturbation of an Essential Pathway: Evaluation of a Glycine Precursor of Coenzyme A

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Supporting Information

ABSTRACT: Pantetheine and its corresponding disulfide pantethine play a key role in metabolism as building blocks of coenzyme A (CoA), an essential cofactor utilized in ∼4% of primary metabolism and central to fatty acid, polyketide, and nonribosomal peptide synthases. Using a combination of recombinant engineering and chemical synthesis, we show that the disulfide of *N*-pantoylglycyl-2-aminoethanethiol (GlyPan), with one fewer carbon than pantetheine, can rescue a mutant *E. coli* strain MG1655Δ*panC* lacking a functional pantothenate synthetase. Using mass spectrometry, we show that the GlyPan variant is accepted by the downstream CoA biosynthetic machinery, ultimately being incorporated into essential acyl carrier proteins. These findings point to further flexibility in CoA-dependent pathways and offer the opportunity to incorporate orthogonal analogues.

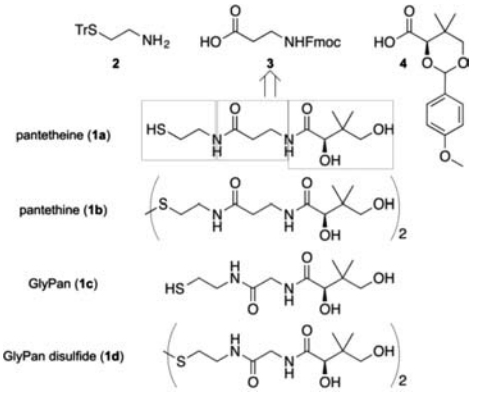

Figure 1. Structures of pantetheine (**1a**), which can be synthesized from components **2**, **3**, and **4**; the disulfide pantethine (**1b**); GlyPan (**1c**); and synthetically prepared GlyPan disulfide (**1d**).

Coenzyme A (CoA) is a critical cofactor found in all organisms, and it has been estimated that ∼4% of all known enzymes use CoA as an obligate cofactor.[1] Its key roles are to serve as a universal acyl carrier and to impart post-translational functionality to fatty acid, polyketide, and nonribosomal peptide biosynthetic carrier proteins.[2] During the latter process, a 4′-phosphopantetheine arm from CoA is transferred to an *apo*-carrier protein (*apo*-CP) by a 4′-phosphopantetheinyl-transferase (PPTase),[3] thereby enabling the resulting *holo*-CP to shuttle acyl or aminoacyl building blocks during assembly.

In 1953, Hoagland and Novelli[4], partially on the basis of evidence from Brown and Snell,[5] confirmed that pantetheine (**1a**) is the biosynthetic precursor to CoA. This discovery has since led to the development of domain-specific synthase probes as well as tools for site-specific labeling of proteins.[6] To date, the bulk of these probes bear functional modifications on their thiol terminus. Only a few studies have examined the functional tolerance within the internal *β*-alanine unit.[7] Interestingly, only a few years after the synthesis of **1a** by Brown and Snell,[8] efforts led by King[9] prepared *N*-pantoylglycyl-2-aminoethanethiol (GlyPan, **1c**; Figure 1) and demonstrated that it is able to support the growth of *Lactobacillus helveticus*, a pantetheine-responsive organism. Remarkably, other than the products of recent synthetic efforts,[7] the observations of King have not been examined in detail.

CoA is produced in *Escherchia coli* through the activity of nine enzymes, beginning with 2-oxoisovalerate (**5**) and passing through 4′-phosphopantetheine (Scheme 1).[10] The first three steps in this pathway can be bypassed by **1a** from media supplementation or catabolic degradation of CoA and fatty acid acyl carrier protein (AcpP). While King's efforts demonstrated that select pantetheine analogues support the growth of pantetheine-responsive organisms, we were instead interested in evaluating their ability to complement pantetheine. In an effort to understand these findings further, we chose to examine carefully the metabolic use of pantetheine analogues varied at the *β*-Ala position with an *E. coli* mutant of CoA biosynthesis lacking pantothenate synthetase (MG1655Δ*panC*, a strain that requires pantetheine supplementation for growth).[11]

In 2004, we reported the development of a modular synthesis of pantethine (**1b**) from three components: *S*-tritylcysteamine (**2**), Fmoc-*β*-Ala (**3**), and protected pantoic acid **4** (Figure 1).[7b]

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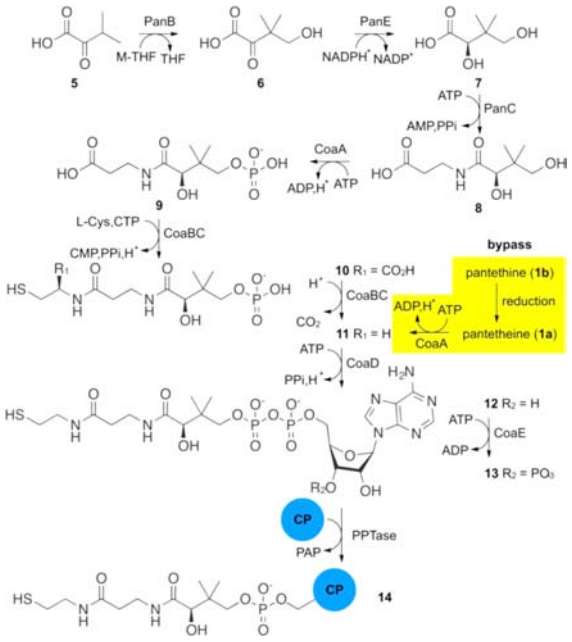
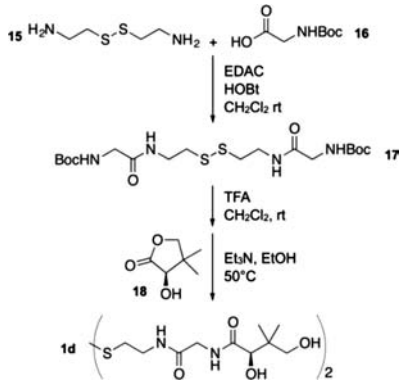
Scheme 1. CoA Biosynthesis in *E. coli* and Its Use to Convert a Carrier Protein (CP) from Its *apo* Form to Its *holo* Form



This route successfully delivered **1b**, which could be reduced in situ to **1a**, in a total of nine steps and an overall yield of 27% (five linear steps). While viable, the length of this route combined with difficulties associated with the thiol-protecting group proved to be problematic when conducted on scales larger than a gram.

In response, we adopted a strategy used in Bristol-Myers Squibb's synthesis of Omapatrilat[12] and focused our efforts on the preparation of disulfide **1d** (Figure 1), thereby eliminating the need for thiol protection. Using a modular design similar to that for **1a**/**1b** (Figure 1), we synthesized **1d** from cystamine (**15**), Boc-Gly (**16**), and D-pantolactone (**18**) (Scheme 2).[9]

Scheme 2. Synthesis of GlyPan Disulfide (1d)



After optimization, we identified conditions for preparing gram quantities of **1d** in two operations. By means of peptide activation, amine **15** and acid **16** were coupled to deliver bisamide **17**. The use of Boc protection allowed us to deprotect **17** and couple the resulting crude bisamine to **18** in warm EtOH buffered with Et_3N. During reaction screening, we found that the use of a sealed flask was the key to the last step, as Et_3N evaporates from the reaction mixture during heating. Using this procedure, we prepared **1d** in 25% overall yield from **15**.

In *E. coli*, absorbed **1b** can bypass CoaBC by in situ reduction followed by conversion to phosphopantetheine (**11**) by a CoaA pantothenate kinase (PanK). This process provides an alternate access to CoA (**13**), as highlighted in Scheme 1.[13] We next examined the ability of **1d** to achieve this bypass. By screening on agar (Figure 2), we found that addition of 1 mM **1d** supports the

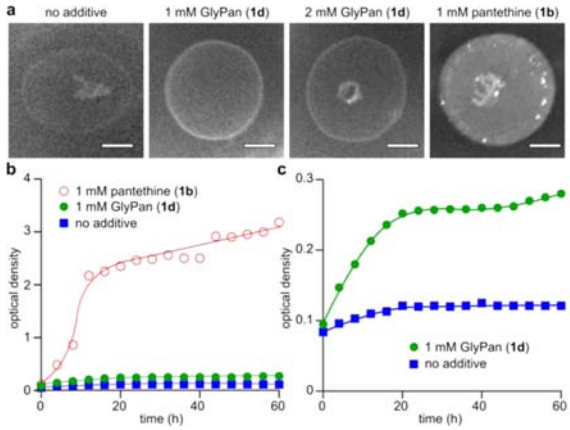


Figure 2. Images depicting the rescue of *E. coli* strain MG1655Δ*panC*. (a) Addition of 1 mM or 2 mM GlyPan (**1d**) rescued the growth of MG1655Δ*panC*, albeit far less efficiently than 1 mM pantethine (**1b**). The images depict a colony of *E. coli* MG1655Δ*panC* grown from a sample spotted on an agar plate. The negative control (no additive) depicts cells spotted on agar lacking **1b** or **1d**. Scale bars denote 1 mm. (b) Addition of 1 mM **1b** or 1 mM **1d** enhanced the growth of the MG1655Δ*panC* mutant in minimal medium. (c) Expansion of the plot in (b) depicting the growth in medium containing 1 mM **1d** versus the no-additive control. Experimental efforts were conducted using disulfides **1b** and **1d**, which were reduced in situ.

growth of *E. coli* MG1655Δ*panC*, a pantothenate auxotroph[11] that can also survive with supplementation of **1b**. While not as effective as **1b**, **1d** was able to enhance the growth of the MG1655Δ*panC E. coli* strain.

We then turned to mass spectral methods to confirm that **1d** enters the primary metabolic pathway. Here we chose to evaluate post-translational modification of AcpP to validate the metabolic elaboration of **1d** (or reduced **1c**) to the CoA analogue and functional utilization of the resulting product. Samples of MG1655Δ*panC E. coli* cells were cultured in M9 minimal medium supplemented with 0.2% glucose, 0.1% casamino acids, 1 mM $MgSO_4$, and 1 mM **1d**. A single colony from an LB plate was used to inoculate 1 mL of supplemented M9 medium. The cells were then grown at 28 °C to confluence (six generations). The starter culture expanded to 10 mL and then 100 mL with an additional three generations per culture (a total of 12 generations of growth from the LB plate). After the cells were harvested by centrifugation, the resulting cell pellet was lysed in phosphate-buffered saline (pH 7.4) via sonication, and the soluble protein fraction was collected after further centrifugation.

Urea-PAGE analysis (Figure 3a) indicated the presence of AcpP in these samples. Using a combination of urea-PAGE and passive elution,[14] we were able to isolate appreciable amounts of proteins, which were subsequently observed by SDS-PAGE (Figure 3b). Using trypsin digestion followed by LC−MS/MS (Figure 3b), we were able to identify the two bands as AcpP and flavodoxin-A (FldA) with 40% and 39% coverage, respectively (Figure 3c). Similarities in the molecular weights and isoelectric points of FldA and AcpP made purification via urea- or SDS-

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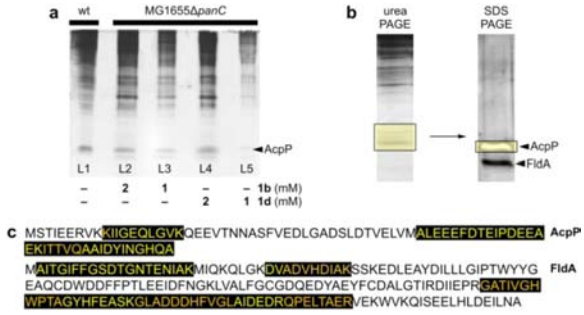


Figure 3. Isolation of GlyPan-modified *holo*-AcpP. (a) Analysis of soluble cell lysate separation via urea-PAGE depicting wild-type *E. coli* (L1) and MG1655Δ*panC E. coli* grown in the presence of 2 mM **1b** (L2), 1 mM **1b** (L3), 2 mM **1d** (L4), and 1 mM **1d** (L5). (b) Urea- and SDS-PAGE gels depicting the purification of AcpP from cell lysates. (c) Protein analysis data indicating the peptides identified in the bands for AcpP and FldA (colored in yellow or orange with black background).

PAGE unfeasible, but the resolution of the LC–MS/MS analysis was sufficient to obtain proteomic data.

Next, we turned to top-down LC–MS/MS methods to characterize these proteins further (Figure 4). We were able to

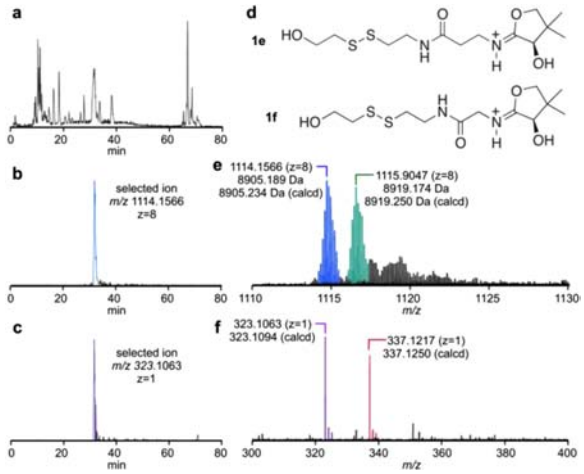
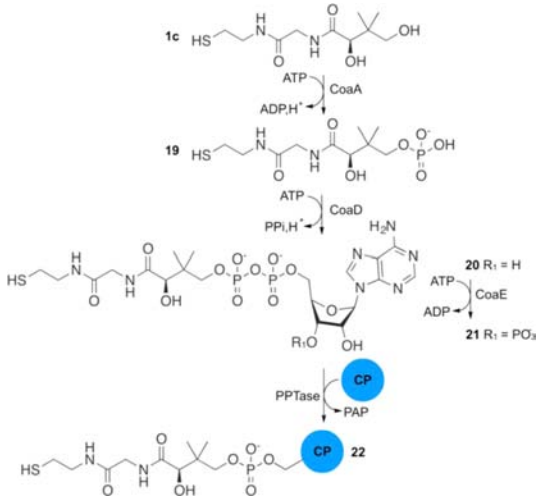


Figure 4. LC–MS/MS evaluation of modified AcpP. (a) Base-peak chromatogram, including selected ions for (b) GlyPan-modified *holo*-AcpP (blue) and (c) GlyPan-ejected ion **1f**. (d) Structures of the 4′-phosphopantetheinyl-ejected ions **1e** and **1f**. (e) The 4′-phosphopantetheinylated AcpPs were also detected at the intact protein level: (blue) GlyPan-loaded AcpP; (green) pantetheine-loaded AcpP. (f) 4′-Phosphopantetheinyl-ejection ions **1e** (red) and **1f** (purple) were detected, indicating the loading of pantetheine (**1a**) and GlyPan (**1c**), respectively. In this experiment, **1a** and **1c** either on *holo*-AcpP or ejected from *holo*-AcpP were detected as *β*-mercaptoethanol (BME) adducts **1e** and **1f**, respectively.

detect the AcpP bearing a *β*-mercaptoethanol (BME) adduct of the GlyPan derivative of 4′-phosphopantetheine (**1f**; Figure 4d) having a mass of 8905.189 Da (expected for $C_{380}H_{612}N_{95}O_{142}PS_3$, 8905.234 Da) as its corresponding $z = 8^+$ ion (blue, Figure 4e). Additionally, we also observed the AcpP bearing the BME adduct of 4′-phosphopantetheine (**1e**; Figure 4d) having a mass of 8919.174 Da (expected for $C_{381}H_{614}N_{95}O_{142}PS_3$, 8919.250 Da) as its corresponding $z = 8^+$ ion (green, Figure 4e).

We further explored the process by examining each enzymatic conversion step (Scheme 3). By using selected combinations of

Scheme 3. Biosynthetic Incorporation of 1c and Its Use to Convert a CP to Its *holo* Form



enzymes in vitro, we were able to demonstrate the selective conversions of **1d** (via **1c**) to **19** and **19** to **20** via LC–MS analyses (Figure 5a). MALDI analyses enabled us also to confirm the full conversion of **19** to the *holo*-GlyPan-modified AcpP **22**.

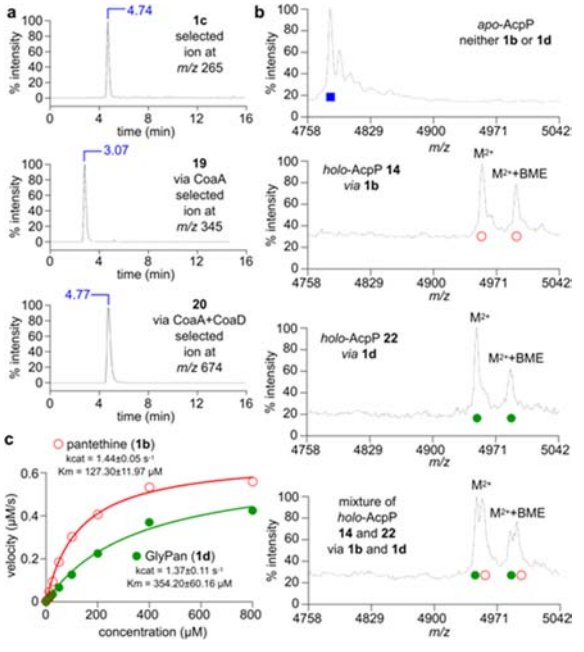


Figure 5. Stepwise evaluation of the biosynthetic uptake of **1d**. (a) LC–MS traces depicting the stepwise conversions of **1d** (via **1c**) to **19** with CoaA and **19** to **20** with CoaD. Reactions were conducted in parallel using combinations of 400 μM **1d**, 0.2 μM CoaA, and 0.2 μM CoaD in 50 mM Tris (pH 8.0) containing 12.5 mM $MgCl_2$ and 8 mM ATP. BME was added at 25 mM prior to MS analysis. (b) Production of *holo*-AcpP **14** (Scheme 1) from **1b** or *holo*-AcpP **22** (Scheme 3) from **1d** was further validated by MALDI analysis. Shown from top to bottom are plots for *apo*-AcpP, **14**, **22**, and a mixture of **14** and **22**. Reactions were conducted using 400 μM **1b** or **1d**, 0.2 μM CoaA, 0.2 μM CoaD, 0.2 μM CoaE, 0.2 μM Sfp, and 50 μM AcpP in 50 mM Tris (pH 8.0) containing 12.5 mM $MgCl_2$ and 8 mM ATP. BME was added at 25 mM prior to MS analysis. (c) Kinetic analyses indicating that the conversion of 12.5–800 μM **1d** to **19** by 0.4 μM PanK is comparable to that obtained using identical conditions with 12.5–800 μM **1b**.

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As shown in Figure 5b, with this method we could distinguish both 4′-phosphopantetheinylated AcpP **14** (Scheme 1) and GlyPan-derived AcpP **22** (Scheme 3). We then applied kinetic analyses to compare the relative rates at which CoaA acted on **1a** and **1c**. Using a coupled assay developed by Strauss and Begley,[15] we determined that PanK is not rate-limiting (Figure 5c).

Finally, we were able to apply tandem mass spectrometry to identify the 4′-phosphopantetheinyl side arm as a source of the 14 amu mass shift.[16] We observed fragment ions from the elimination of BME adducts **1e** (red, Figure 4f) and **1f** (purple, Figure 4f), confirming the loading of **1c** onto AcpP. This experiment also indicated that the original pantetheine arm was still present in the bacteria, although ∼12 generations had been passed since they were grown in medium supplemented with **1b**. At optimal usage, bacteria containing 1 mM **1b** at generation 1 would yield progeny with 0.25 μM **1b** at generation 12. While **1b** was detected after many generations, it was clear that without the addition of **1d** or **1b**, bacterial growth was held at a minimum. More importantly, the fact that **1d** was observed on proteins in bacterial cells whose growth was enhanced upon the addition of **1d** supports the acceptance of **1d** by the *E. coli* fatty acid machinery. Taken together, these data indicate that GlyPan (**1c**), formed from **1d**, is effectively incorporated into CoA biosynthesis and used for post-translational modification of *E. coli* fatty acid carrier proteins.

Overall, these studies reveal the ability of a strain of *E. coli* that is deficient in its ability to produce an essential and ubiquitous cofactor to accept a modified analogue of pantetheine, GlyPan (**1c**). The nature of the primary functions of CoA in prokaryotic cellular biology makes this substitution intriguing. For its role in fatty acid synthesis, the CoA analogue from **1c** having one fewer carbon must interact with at least nine catalytic enzymes during condensation and reduction steps in addition to acyltransferase enzymes that guide newly synthesized lipids to their respective pathways. The number of transformations that are able to proceed despite the change in the identity of the 4′-phosphopantetheine side arm cannot be overlooked. Consideration of this substitution must include not only the acceptance of **1c** into CoA biosynthetic enzymes but also the promiscuity of all of the enzymes that perform downstream biosynthesis and regulatory functions. The fact that **1a** remained within bacterial cells in media supplemented with **1d** and lacking **1b** poses further questions about pantetheine metabolism, therein suggesting additional biosynthetic and metabolic implications (see the further discussion in the Supporting Information).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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