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Metabolic perturbation of an essential pathway: evaluation of a glycine precursor of Coenzyme A

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Abstract

Pantetheine, and its corresponding disulfide pantethine, play a key role in metabolism as a building block of coenzyme A (CoA), an essential cofactor utilized in ~4% of primary metabolism and central to fatty acid, polyketide, and non-ribosomal peptide synthases. Using a combination of recombinant engineering and chemical synthesis, we demonstrate that the disulfide of *N*-pantoylglycyl-2-aminoethanethiol (GlyPan), with one carbon deletion from pantetheine, can rescue a mutant *E. coli* strain MG1655Δ*panC* lacking a functional pantothenate synthetase. Using mass spectral methods, we demonstrate 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 analogs.

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 as a universal acyl carrier and to impart post-translational functionality to fatty acid, polyketide and non-ribosomal 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 Novelli⁴ confirmed that pantetheine (**1a**), based in part by evidence from Brown and Snell,⁵ was the biosynthetic precursor to CoA. This discovery has since led to the development of domain specific synthase probes as well as tools for the 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 Brown and Snell's synthesis of **1a**,⁸ efforts led by King⁹ prepared *N*-pantoylglycyl-2-aminoethanethiol (GlyPan, **1c**, Fig. 1) and demonstrated that it was able to support the growth of *Lactobacillus helveticus*, a

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Synthetic methods and spectral data for the preparation of **1c**, as well as detailed biochemical and mass spectroscopic procedures for all experiments described within is available free of charge via the Internet at <http://pubs.acs.org>.

pantetheine-responsive organism. Remarkably, other than products of recent synthetic efforts,⁷ the observations of King have not been examined in detail.

CoA is produced in *E. coli* through the activity of nine enzymes, beginning with 2-oxoisovalerate (**5**, Scheme 1) and passing through 4'-phosphopantetheine.¹⁰ The first three steps in this pathway can be bypassed by pantetheine from media supplementation or catabolic degradation of CoA and the fatty acid acyl carrier protein (AcpP). While King's efforts demonstrated that select pantetheine analogs support the growth of pantetheine-responsive organisms, we were instead interested in evaluating their ability to complement pantetheine. In an effort to further understand these findings, we chose to carefully examine the metabolic use of pantetheine analogs varied at the β -Ala position with an *E. coli* mutant of CoA biosynthesis lacking pantothenate synthetase (MG1655 Δ panC,¹¹ a strain that requires pantetheine supplementation for growth).¹²

In 2004, we reported on the development of a modular synthesis of pantethine (**1b**) from three components: *S*-tritylcysteamine (**2**), Fmoc- β -Ala (**3**), and protected pantoic acid **4** (Fig. 1).^{7b} This route successfully delivered **1b**, which could be reduced *in situ* to pantetheine (**1a**), in 9 total 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 problematic when conducted at scales larger than a gram.

In response, we adopted a strategy used in Bristol-Myers Squibb's synthesis of Omapatrilat¹¹ and focused our efforts towards the preparation of disulfide **1d** (Fig. 1), thereby eliminating the need for thiol protection. Using a comparable modular design as in our preparation of **1a/1b** (Fig. 1), the synthesis of **1d** (Scheme 1) was derived from cystamine (**14**), Boc-Gly (**15**), and D-pantolactone (**17**).^{9a}

After optimization, conditions were identified to prepare gram quantities of **1d** in two operations. Using peptide activation, amine **14** and acid **15** were coupled to deliver *bis*-amide **16**. By using Boc protection, we could deprotect **6** and coupled the resulting crude *bis*-amine to **17** in warm EtOH buffered with Et₃N. During reaction screening, we found that use of a sealed flask was key to the last step, as Et₃N evaporates from the reaction mixture during heating. Using this procedure, we were able to prepare **1d** in 25% overall yield from **14**.

In *E. coli*, absorbed pantethine (**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 turned to examine the ability of the GlyPan disulfide (**1d**) to achieve this bypass. By screening on agar (Fig. 2), we found that addition of 1 mM **1d** supports growth of *E. coli* MG1655 Δ panC, a pantothenate auxotroph¹² that can also survive with supplementation of pantethine (**1b**). While not as effective as **1b**, **1d** was able to enhance growth of the MG1655 Δ panC *E. coli* strain.

We then turned to mass spectral methods to confirm that the GlyPan disulfide (**1d**) entered the primary metabolic pathway. Here we chose to evaluate post-translational modification of AcpP in order to validate metabolic elaboration of **1d** (or reduced **1c**) to the CoA analog and functional utilization of the resulting product. Samples of MG1655 Δ panC *E. coli* cells were cultured in M9 minimal media supplemented with 0.2% glucose, 0.1% casamino acids, 1 mM MgSO₄ and 1 mM GlyPan disulfide (**1d**). A single colony from an LB plate was used to inoculate 1 mL of supplemented M9 media. The cells were then grown at 28 °C to confluence (6 generations). The starter culture expanded to 10 mL and then 100 mL with an additional 3 generations per culture (a total of 12 generations of growth from the LB plate).

The cells were harvested by centrifugation, and the resulting cell pellet was lysed in PBS buffer pH 7.4 via sonication, and the soluble protein fraction was collected after further centrifugation.

Urea-PAGE analysis (Fig. 3a) indicated the presence of fatty acid acyl carrier protein, AcpP, in these samples. Using a combination of urea-PAGE and passive elution,¹⁴ we were able to isolate proteins in appreciable amounts, which were subsequently observed by SDS-PAGE (Fig. 3b). Using trypsin-digestion followed by LC-MS-MS analyses (Fig. 3b), we were able to identify the two bands as AcpP and flavodoxin-A (FldA) with 40% and 39% coverage, respectively (Fig. 3c). Similarities in the molecular weight and isoelectric point of FldA and AcpP made purification via urea-PAGE or SDS-PAGE unfeasible, but sufficient resolution was obtained during LC-MS/MS analysis to obtain proteomic data.

Next, we turned to top-down LC-MS/MS methods to further characterize these proteins (Fig. 4). We were able to detect the AcpP bearing a β -mercaptoethanol (BME) adduct of the GlyPan derivative of 4'-phosphopantetheine (**1f**, Fig. 4d) with an ion m/z of 8905.189 Da (expected m/z of 8905.234 Da for $C_{380}H_{612}N_{95}O_{142}PS_3$), as its corresponding $z=8^+$ ion (blue, Fig. 4d). Additionally, we also observed adducts of AcpP bearing the BME adduct of 4'-phosphopantetheine (**1e**, Fig. 4d), as given by m/z of 8919.174 Da (expected m/z of 8919.250 Da for $C_{381}H_{614}N_{95}O_{142}PS_3$) from its corresponding $z=8^+$ ion (green, Fig. 4d).

We further explored the process by examining each step of the enzymatic conversions, as shown in Scheme 3. By using select combinations of enzymes *in vitro*, we were able to demonstrate the selective conversion of GlyPan (**1d**) via **1c** to **19** and **19** to **20** via LC-MS analyses (Fig. 5a). Combined with MALDI analyses, we were also able to further confirm the full conversion of **19** to the *holo*-GlyPan modified AcpP **22**. As shown in Fig. 5b, we were able to use this method to distinguish both the 4'-phosphopantetheinylated AcpP (**14**, Scheme 1) and GlyPan-derived AcpP (**22**, Scheme 3). We then applied kinetic analyses to compare the relative rates in which CoaA acted on **1a** and **1c**. Using a coupled assay developed by Strauss and Begley,¹⁵ we determine that PanK was not rate limiting (Fig. 5c).

Finally, we were able to apply tandem mass spectrometry to identify the 4'-phosphopantetheinyl sidearm as a source of the 14 amu mass shift.¹⁶ Using this method, we observed fragment ions from the elimination of β -mercaptoethanol adducts **1e** (red, Fig. 4f) and **1f** (purple, Fig. 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 media supplemented with pantetheine (**1b**). At optimal usage, bacteria containing 1 mM **1b** at generation 1 would yield progeny with 0.25 μ M **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 GlyPan disulfide (**1d**) was observed on proteins in bacterial cells whose growth was enhanced upon the addition of **1d** supports acceptance of **1d** by *E. coli* fatty acid machinery. Taken together, these data indicate that GlyPan (**1c**), via **1d**, is effectively incorporated into CoA biosynthesis and used to post-translationally modify *E. coli* fatty acid carrier proteins.

Overall these studies reveal the ability of a strain of *E. coli*, deficient in its ability to produce an essential and ubiquitous cofactor, to accept a modified analog 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 corresponding one carbon reduced CoA analog from **1c** must interact with at least nine catalytic enzymes during condensation and reduction steps in addition to acyltransferase enzymes that guide newly synthe-sized lipids to their respective pathways.

The number of transformations that are able to proceed despite the change in the identity of the 4'-phosphopantetheine sidearm cannot be overlooked. When considering this substitution, one must consider not only the acceptance of **1c** into CoA biosynthetic enzymes, but also the promiscuity of all enzymes that perform downstream biosynthesis and regulatory functions. The fact that pantetheine (**1a**) remained within the bacterial cells in media supplemented with GlyPan disulfide (**1d**) and was not supplemented with **1b** poses further questions about pantetheine metabolism, therein suggesting additional biosynthetic and metabolic implications (see further discussion in the Supporting Information).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

Figure 2.

Images depicting rescue of *E. coli* strain MG1655 Δ *panC*. **a)** Addition of 1 mM or 2 mM GlyPan (**1d**) rescued growth of MG1655 Δ *panC*, however, this rescue was far less efficient than 1 mM pantethine (**1b**). Images depict a colony of *E. coli* MG1655 Δ *panC* grown from a sample spotted on an agar plate. Negative control (no additive) depicts cells spotted on agar lacking **1b** or **1d**. Bars denote 1 mm. **b)** Addition of 1 mM **1b** or 1 mM **1d** enhances the growth of the MG1655 Δ *panC* mutant in minimal media. **c)** Expansion of the plot in b) depicting the growth in media containing 1 mM GlyPan (**1d**) *versus* the no additive control. Experimental efforts were conducted using disulfides **1b** and **1d**, which were reduced *in situ*.

Figure 3.

Isolation of GlyPan modified *holo*-AcpP. **(a)** Analysis of soluble cell lysate separation via urea-PAGE depicting wild type *E. coli* (L1), 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 ID analysis data indicating peptides identified in the bands for AcpP and FldA colored in yellow or orange with black background.

Figure 4.

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

Figure 5.

Stepwise evaluation of the biosynthetic uptake of GlyPan (**1d**). **a**) LC/MS traces depicting the stepwise conversion of GlyPan (**1c**) via **1d** to **19** with CoaA, and **20** with CoaA and 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. β -mercaptoethanol (BME) was added to 25 mM prior to MS analysis. **b**) The production of holo-AcpP **14** (Scheme 1) via **1b** or holo-AcpP **22** (Scheme 3) via **1d** was further validated by MALDI analysis. The top three plots denote the apo-AcpP, holo-AcpP **14**, holo-AcpP **22** and a mixture of holo-AcpPs **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 to 25 mM prior to MS analysis. **c**) Kinetic analyses indicate 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**.

Scheme 1.

CoA biosynthesis in *E. coli* and its use to convert a carrier protein (CP) from its *apo*- to *holo*-form.

Scheme 2.
Synthesis of GlyPan disulfide **1d**.

Scheme 3.

Biosynthetic incorporation of GlyPan (**1c**) and its use to convert a carrier protein (CP) to its *holo*-form.