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Mechanism of regulation of pantothenate biosynthesis by the PanD-PanZ.AcCoA complex reveals an additional mode of action for the antimetabolite N-pentyl pantothenamide (N5-Pan)

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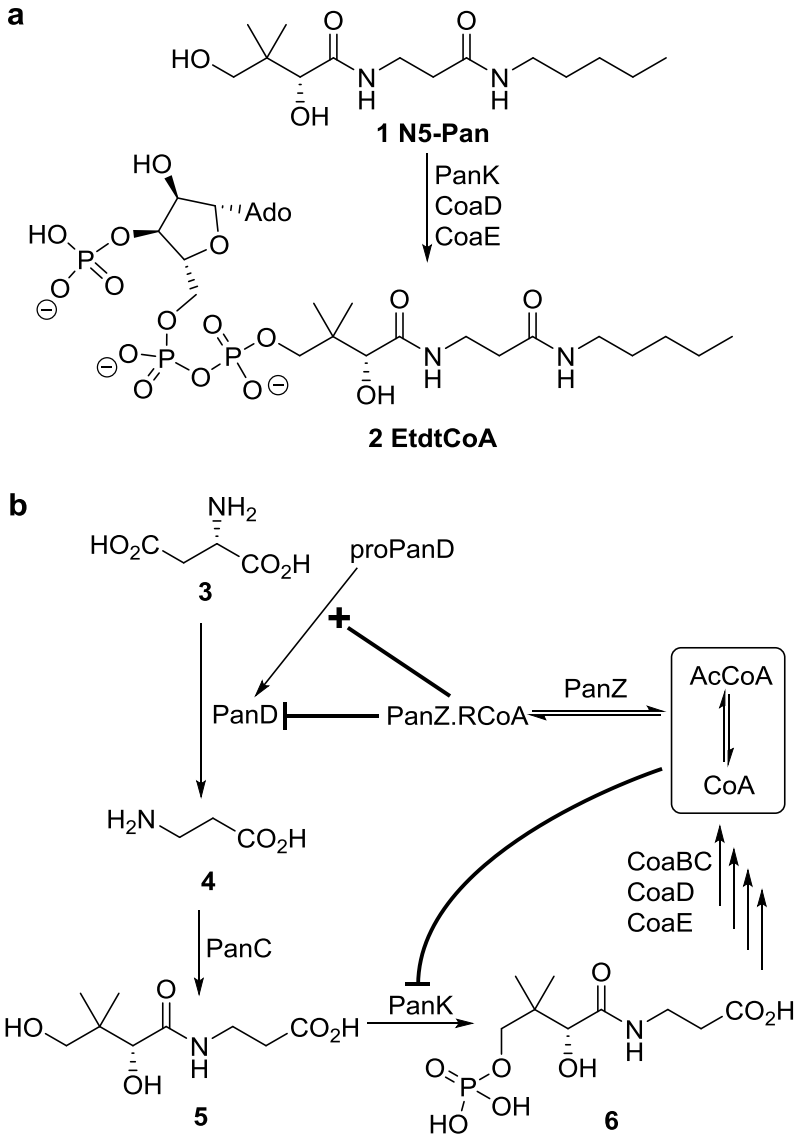
KEYWORDS Metabolic regulation, antimetabolites, antibiotics, vitamin biosynthesis, pyruvoyl-dependent enzymes

ABSTRACT The antimetabolite pentyl pantothenamide has broad spectrum antibiotic activity but exhibits enhanced activity against *Escherichia coli*. The PanDZ complex has been proposed to regulate the pantothenate biosynthetic pathway in *E. coli* by limiting the supply of β-alanine in response to coenzyme A concentration. We show that formation of this complex between activated aspartate decarboxylase (PanD) and PanZ leads to sequestration of the pyruvoyl cofactor as a ketone hydrate and demonstrate that both PanZ overexpression-linked β-alanine auxotrophy and pentyl pantothenamide toxicity are due to formation of this complex. This both demonstrates that the PanDZ complex regulates pantothenate biosynthesis in a cellular context and validates the complex as a target for antibiotic development.

INTRODUCTION

The antimetabolite pentyl pantothenamide **1** (N5-Pan, scheme 1a) was first described in 1970[1]. Like other pantothenamides it has broad spectrum antibiotic activity but, uniquely, shows an order of magnitude improvement against *Escherichia coli*, with a minimum inhibitory concentration (MIC) of 2 μg mL^{-1}. Subsequent studies by Strauss and Begley demonstrated it is metabolized by three enzymes from the CoA biosynthesis pathway (pantothenate kinase (PanK), pantetheine adenylyltransferase (CoaD) and dephosphocoenzyme A kinase(CoaE)) to form ethyldethiacoenzyme A (EtdtCoA, **2**)[2]. Remarkably the *E. coli* metabolic enzymes favor the antimetabolite over the natural substrates by a factor of more than 10-fold. EtdtCoA is subsequently used as a substrate by phosphopantetheinyl transferases, forming inactive acyl carrier proteins (*crypto*-ACPs)[3] as well as poisoning the cellular pool of coenzyme A. A similar mechanism of inhibition is observed in other bacteria, depending on the subtype of pantothenate kinase (PanK) present in the organism[4]. Only promiscuous $PanK_I$ and $PanK_{II}$ type enzymes

which accept pantetheine as an alternative substrate to pantothenate are able to metabolise pentyl pantothenamide[5]. $PanK_{III}$-encoding organisms are resistant to the antimetabolite[6]. Formation of *crypto*-ACPs is not, however, the only source of toxicity in *E. coli*. Thomas and Cronan showed that *crypto*-ACPs can be effectively recycled by the action of ACP hydrolase[7] and, in the presence of an exogenous supply of pantothenate, the cells are rescued from growth inhibition. They therefore proposed that the toxic effects of pentyl pantothenamide were due to depletion of the cellular coenzyme A pool by an unknown additional mechanism.



Scheme 1. **Relationship between pentyl pantothenamide (N5-Pan) and regulation of pantothenate biosynthesis**. **a** N5-Pan **1** is metabolized by PanK, CoaD and CoaE to generate ethyl dethiacoenzyme A (EtdtCoA, **2**). **b** Pathway from L-aspartate to coenzyme A. β-Alanine **4** is produced by decarboxylation of L-aspartate **3** by aspartate α-decarboxylase (PanD). β-Alanine then forms pantothenate **5** which is subsequently metabolized by PanK, CoaB, CoaC, CoaD and CoaE to form coenzyme A **3**. PanD is produced as a zymogen (proPanD) which is activated by the PanZ.RCoA complex but is also inhibited by the same complex[8]. (RCoA = AcCoA or CoA)

We have recently reported the structure of the complex formed between the zymogen of *E. coli* aspartate α-decarboxylase (proPanD) and its regulatory protein, PanZ[8]. PanD is responsible for the production of β-alanine in the pantothenate biosynthesis pathway[9] and its catalytic action is dependent upon formation of a covalently-bound pyruvoyl group from a serine residue via rearrangement of its peptide backbone[10]. PanZ is required for the *in vivo* activation of *E. coli* proPanD, as the uncatalysed rearrangement is too slow to support growth. PanZ is only found in a limited subset of enteric γ-proteobacteria, including the pathogens *Salmonella enterica*[11] and *Yersinia pestis*[12]. In those organisms which do not encode PanZ, such as *Mycobacterium tuberculosis*[13], it appears that the rearrangement is faster and autocatalytic. Our biophysical[14] and structural[8] characterization of the *E. coli* protein-protein complex revealed that the protein-protein interaction between PanZ and proPanD is dependent upon the presence of coenzyme A or acetyl CoA (AcCoA). Following this observation, we demonstrated that PanZ has a second inhibitory role. While low-level expression of PanZ relieves the β-alanine auxotrophy caused by *panZ* deletion, overexpression of PanZ leads to inhibition of the pantothenate biosynthesis pathway due to inhibition of catalysis by activated PanD[8]. At physiological concentrations of PanZ, we hypothesized that the protein-protein interaction provides a negative feedback

mechanism for the pantothenate biosynthesis pathway in response to cellular CoA concentration (scheme 1b). Given this regulatory mechanism, we investigated whether the enhanced toxicity of pentyl pantothenamide is due to accumulation of EtdtCoA, leading to down-regulation of pantothenate biosynthesis mediated by this complex.

EXPERIMENTAL METHODS

Construction of chromosomal *E. coli panD*(K119A) and other mutants The 3.4 kb *ClaI*-*SalI* fragment encompassing the *panD* gene from the chromosome of *E. coli* MG1655 was cloned into the *ClaI*-*SalI* site of pBR322 to construct pBR322*panD*. pBR322*panD* was *ClaI*-digested and the consequent 5'-overhang was filled by the Klenow fragment of DNA Polymerase I to form a blunt end; the linearized pBR322*panD* was further digested by *SalI*. Similarly, pKH5002SB[15] was digested with *XbaI* and the 5' overhang filled by the Klenow fragment of PolI before being further digested by *SalI*. The *panD*-containing part of the *ClaI*(blunt)-*SalI* fragment from pBR322*panD* was cloned into the *XbaI*(blunt)-*SalI* site of pKH5002SB, resulting in pKH5002SB*panD*. The K119A mutation of *panD* (*panD*(K119A)) was introduced *in vitro* by overlap extension PCR[16] using primer sets [panDmutU and panD(K119A)L] and [panD(K119A)U and panDmutL] with pBR322*panD* as a template, to generate a 0.77 kb NsiI-AflII fragment containing the *panD* gene. To construct pKH5002SB*panD*(K119A), the *NsiI*-*AflII* fragment with the *panD*(K119A) mutation was exchanged with the corresponding wild-type fragment of pKH5002SB*panD*. pKH5002SB*panD*(K119A) was transformed into *E. coli* MG1655. Ampicillin-resistant clones, in which pKH5002SB*panD*(K119A) was integrated into the chromosome by homologous recombination, were obtained because the origin of the vector has a mutation such that the vector can be replicated only in *rnhA* mutant strains. pKH5002SB encodes the *sacB* gene of *Bacillus subtilis*, which is lethal to *E. coli* cells in the presence of

sucrose. The transformants were therefore spread on sucrose-containing plates to select those colonies which lost the wild-type *panD* gene (or *panD*(K119A) gene) as well as the vector sequence by a second homologous recombination. The mutation was confirmed by PCR and DNA sequencing. *panD*(K14A), *panD*(K53A) and *panD*(K115A) were constructed using the same method, except that the primers [panD(K14A)U and panD(K14A)L], [panD(K53A)U and panD(K53A)L] and [panD(K115A)U and panD(K115A)L], respectively, were used. (See supplementary information)

Construction of pBAD24*panZ* mutants The panZ(R73A) mutation was introduced in vitro by overlap extension PCR using primer sets [panZBADU40 and panZ(R73A)L] and [panZ(R73A)U and panZL3] using MG1655 genomic DNA as a template. The PCR product was digested by EcoRI and SphI and cloned into the EcoRI-SphI sites of pBAD24. pBAD24panZ(R43A), pBAD24panZ(N45A) and pBAD24panZ(T72A) were constructed similarly using the primers listed in supplementary information.

Overexpression and purification of proteins All proteins were overexpressed as described previously[8,12,17]. For crystallization, PanD(WT) was overexpressed from vector pRSETApanD in E. coli C41(DE3) cells[17]. For SEC analysis, PanD(WT) and PanD(K119A) were overexpressed from pET28apanD in E. coli *Δ*panD *Δ*panZ cells[12]. For crystallization and ITC analysis, PanZ(WT) was overexpressed using vector pET28apanZ in E. coli *Δ*panD *Δ*panZ (DE3) cells[8]. For SEC analysis, PanZ(WT) was overexpressed using vector pBAD24panZ in E. coli *Δ*panD *Δ*panZ cells. For SEC and ITC analysis, PanZ(R73A) was overexpressed using pBAD24panZ(R73A) in E. coli *Δ*panD *Δ*panZ cells. All proteins were purified by sequential immobilized metal-affinity chromatography and size-exclusion chromatography as described

previously[8]. CoaD and CoaE were overexpressed using the expression clones from the Aska clones collection[26] and purified by single-step immobilized metal-affinity chromatography.

Crystallization and structural determination For structural studies, the final size-exclusion chromatographic step for PanD and PanZ was carried out with isocratic elution with Tris buffer (50 mM, 100 mM NaCl, 0.1 mM DTT, pH 7.5). The proteins were mixed in a 10:11 PanD:PanZ ratio (protomer to monomer), concentrated to ~9 mg mL^{-1} (Amicon centrifugal concentrator 10 kDa MWCO, 4,500 g) and 2 equivalent of AcCoA added.

Crystallisation Bipyramidal crystals were obtained by hanging drop vapour diffusion method in 24-well plates. The protein was crystallised in 200 mM KSCN, 100 mM Bis-Tris propane pH 6.5, 20% *v/v* PEG 3350 at 18 °C. Crystal size varied depending on protein:mother liquor ratios of the drops. 4 μL drops (3 μL protein+1 μL mother liquor) gave the largest, best-diffracting crystals.

Data collection Crystals were cryo-protected stepwise in mother liquor containing 5%, 10% and 20% *v/v* glycerol by soaking for a few seconds in each condition before flash-cooling in liquid nitrogen. Diffraction data were collected at 100 K under a cryo-stream of dry nitrogen at beamline I03 (Diamond Light Source) at λ= 0.9763 Å. 900 frames of 0.2° oscillation, 0.1 s exposure and 30% transmission were collected to a maximum resolution of 1.16 Å.

Data reduction, structure solution and refinement The data were integrated in space group I4 (a=b=85.9 Å, c=80.1 Å, α=β=γ=90°) using XDS[18]. Data were scaled and merged in Aimless[19]. The structure was solved by molecular replacement of the PDB 4CRY model using Molrep[20] and was iteratively manually rebuilt and refined with a mixed isotropic and anisotropic B factor model using Coot[21] and Refmac5[22] respectively.

Table 1 Crystallographic processing and refinement statistics Numbers in parentheses refer to highest resolution shell.

	WT PanD-PanZ.AcCoA
Beamline	Diamond IO3
Temperature	100 K
Space group	I4
Cell dimensions a, b, c (Å)	85.9, 85.9, 80.1
Resolution (Å)	29.28-1.16 (1.18-1.16)
R_{merge} (%)	7.7 (56.7)
$R_{p.i.m.}$ (%)	5.4 (39.5)
$<I> / \sigma I$	9.0 (2.1)
Completeness (%)	99.6 (93.7)
Redundancy	5.1 (4.4)
Refinement	
PDB ID	5LS7
Resolution (Å)	29.28 -1.16 (1.19-1.16)
No. reflections	99822 (6688)
No. free reflections	4966 (391)
R_{work} (%)	11.3 (19.6)
R_{free} (%)	13.7 (22.3)
No. atoms	
Protein	2130
Ligand/ion	82
Water	294
Average B-factors	
Protein (Å^2)	16.96
Ligand/ion (Å^2)	19.04
Water (Å^2)	31.69
R.m.s. deviations	
Bond lengths (Å)	0.034
Bond angles (°)	2.85

Isothermal titration calorimetry All proteins for ITC were purified by size-exclusion chromatography into 50 mM Tris, 100 mM NaCl, 0.1 mM DTT, pH 7.4. Proteins were concentrated by centrifugal concentration (Amicon 10 kDa MWCO). Stock solutions of AcCoA were prepared at 1 or 5 mM concentration in gel filtration buffer and diluted to working concentrations using the flowthrough from centrifugal concentration. Binding assays were

carried out by isothermal titration calorimetry (ITC) using a Microcal iTC200 (GE) thermostatted at 25 °C. The ligand sample was loaded into the sample cell (200 μL) and the titrant was loaded into the sample syringe (70 μL). Each titration experiment consisted of a sacrificial injection of 0.4 μL followed by 19 injections of 2 μL. Titration data were analysed using NITPIC[23] and globally fitted in SEDPHAT[24].

Synthesis of N-pentyl pantothenamide (N5-Pan, 1) and Ethyldethiacoenzyme A (EtdtCoA, 2) Pentyl pantothenamide was synthesized via the route of Strauss and Begley[2] and purified by flash-column chromatography. EtdtCoA was generated via adaptation of literature procedures[2,25]. N-pentyl 4-dibenzylphosphopantothenamide was generated via phosphorylation of N5-Pan, **1** using N-diethyl dibenzylphosphoramidate before deprotection by catalytic hydrogenation. EtdtCoA was generated via enzymatic turnover using CoaD and CoaE (overexpressed using constructs from the Aska clone collection[26]) and purified by sequential reverse-phase HPLC and desalting steps. See supplementary information for full synthetic procedures.

Growth assay of *E. coli* strains For assay on solid media, cell strains were grown to mid-log phase in L or LB media before isolation of cells by centrifugation, washing three times in an equal volume of 1 × M9 media and resuspension in the original volume of M9 media. Cell optical density (600 nm) was used to quantify cell density prior to preparation of cell dilutions at OD_{600} 0.01, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. 2 μL samples of each dilution were plated onto selective media and grown for 45-48 hours. For continuous growth assay in liquid culture, exponentially growing cells in L media were washed 3 times with M9 glucose minimal media before dilution to a final density of 10^{-4} in 3 mL defined growth media, cell growth was monitored using a Bio-Photorecorder TVS062CA (Advantec) with incubation at 37 ºC. For end-point liquid culture

assays exponentially growing cells in 1% tryptone (or M9 glucose) at 37 ºC were diluted to a final concentration of 10^{-4} in a total volume of 110 μL and incubated overnight at 37 ºC for 24 h.

RESULTS

Our previous studies of the system were focused on the mechanism of PanD activation, and we therefore determined the structure of an inactivatable PanD(T57V) site-directed mutant[28] bound to PanZ.AcCoA. This structural analysis, together with independent evidence from both isothermal titration calorimetry and NMR, demonstrated that either coenzyme A or acetyl coenzyme A was essential for the interaction of the pair of proteins. Binding of coenzyme A or its derivatives appears to structure the P loop of PanZ, enabling it to form a tight interaction with the C-terminal peptide of PanD(T57V). Our hypothesis that PanZ regulates PanD in addition to catalyzing conversion of proPanD to PanD was based upon two observations: inhibition of PanD activity *in vitro* by PanZ and β-alanine auxotrophy as a result of overexpression of PanZ. Addition of PanZ.AcCoA to a PanD activity assay showed a reduction in PanD enzymatic activity in both NMR and ITC-based assays. Confirmation of the regulatory mechanism in a cellular context requires additional proof; that PanZ interacts with PanD in a CoA-dependent manner; that CoA concentration controls this interaction in the cell; that the interaction between PanZ and PanD is required for growth inhibition; and that the mechanism occurs at native-levels of protein expression.

Structural characterisation of the PanD-PanZ complex We initially used X-ray crystallography to confirm that the interaction of PanZ.AcCoA with the activated wild-type enzyme is the same as that with the zymogen and CoA-dependent. Partially-activated PanD (purified from a *panZ*$^+$ strain of *E. coli* and therefore isolated as a mixture of both the proPanD zymogen and the

catalytically-active PanD forms[10a]) was mixed with PanZ.AcCoA in a 1:1 ratio, crystals of the complex were obtained, diffraction data collected to 1.16 Å and the structure determined by molecular replacement (see table 1). The overall architecture of the protein complex is isostructural with that observed for the PanD(T57V)-PanZ complex (see figure S1)[8]. Four molecules of PanZ bind symmetrically to each face of the PanD tetramer and AcCoA structures the P-loop of PanZ enabling interaction with the C-terminus of PanD[8].

A mixture of the proPanD and PanD was used in crystallization trials, but the structure in the crystal is that of the fully activated PanD, consistent with activation by PanZ during the crystallization process. The electron density at the active site is, however, inconsistent with the presence of a pyruvoyl group as observed in the *apo*-enzyme by Albert *et al.* (see figure S2).[10b] Instead a tetrahedral structure is present, consistent with the presence of the (usually) thermodynamically-disfavoured ketone hydrate (Figure 1a). We repeated the structural elucidation using fully activated PanD. We observed the same structure using both room-temperature and cryo-cooled crystals (data not shown) indicating that the hydrate is formed from the pyruvoyl cofactor and is not an intermediate in the activation reaction. This state is stabilized by a hydrogen-bond to the amide of Gly24, which is held in place due to interactions with PanZ (Figure 1c). This suggests that binding of PanZ to the protein is able to generate specific changes in the microenvironment of the active site favouring this state. This provides a rationale for inhibition of catalysis by PanZ – in order to bind to substrate, the protein-protein complex must first dissociate allowing the pyruvoyl group to reform – substrate cannot bind at all in the presence of the inhibitory protein.

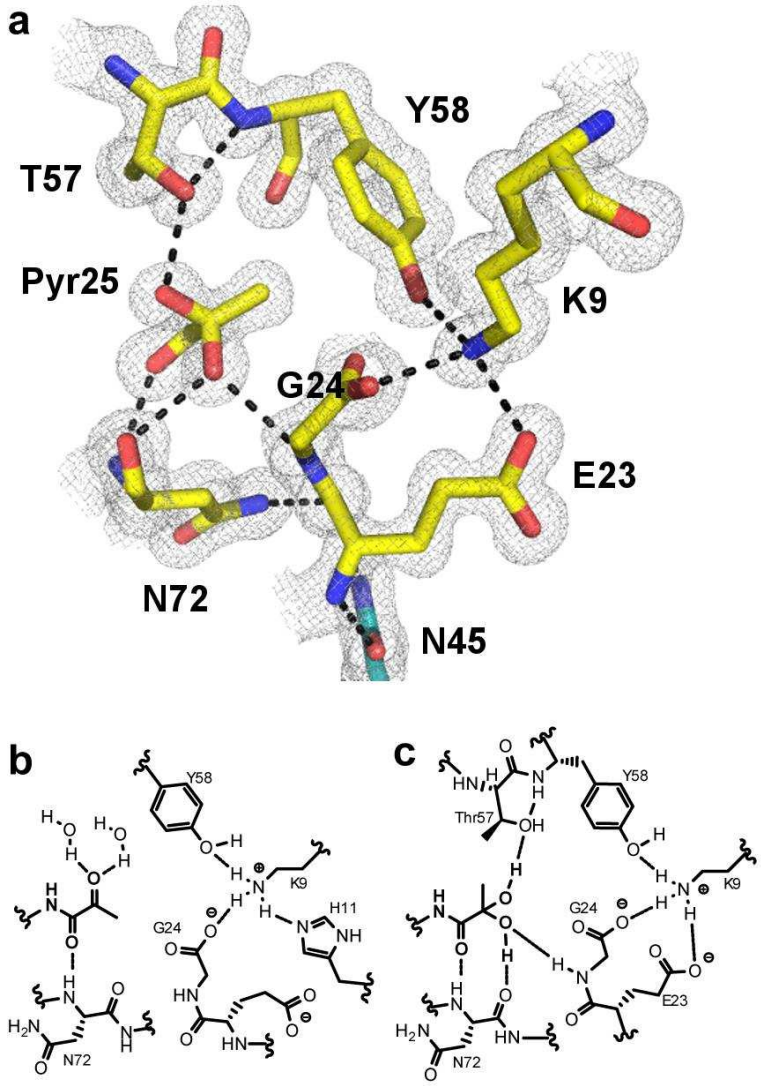


Figure 1. a Crystal structure of the PanZ.AcCoA-ADC complex at 1.16 Å resolution reveals the pyruvoyl cofactor of activated ADC is present as a ketone hydrate in the complex. 2Fo-Fc electron density is shown contoured at 1 rmsd as grey mesh. ADC carbons are shown in yellow, PanZ carbons in cyan (limited to residue N45, bottom), figure generated using PyMol. **b** Hydrogen-bonding interactions in the vicinity of the pyruvoyl group in the *apo*-state (1aw8, Albert et al.[10b]). The pyruvoyl keto-group forms hydrogen bonds to solvent molecules. Residue K9 forms hydrogen bonds to Y58, H11 and the carboxylate of G24 (formed as a result of the activation reaction). **c** Hydrogen-bonding interactions in PanZ.AcCoA-ADC complex (5ls7, this

work). A methyl-ketone hydrate form of the pyruvoyl cofactor is stabilized by hydrogen-bonds to G24, T57 and N72. Binding of PanZ to the surface of PanD leads to formation of a hydrogen-bond between PanZ-N45 and the backbone carbonyl of E23 distorting the hydrogen-bonding network in the active site and displacing H11 from binding to K9.

Overexpression linked growth inhibition is dependent upon CoA-dependent interaction of PanZ with PanD The initial *in vivo* evidence for PanZ-induced inhibition of catalysis by ADC was that, while uninduced, leaky expression of PanZ leads to functional complementation of a *ΔpanZ* strain of *E. coli*[12], *over*expression does not[8]. We tested for this phenotype in the *panZ*$^{+}$ *E. coli* MG1655 strain. Overexpression of PanZ restricts bacterial growth on minimal media by inducing β-alanine auxotrophy (figure 2a). It has previously been shown that *B. subtilis* ADC does not require PanZ for activation – an *E. coli ΔpanD*::*BspanD ΔpanZ* strain in which the *E. coli* ADC is replaced with the Bacillus protein is able to grow without β-alanine supplementation[12]. Furthermore, interaction of *E. coli* ADC and PanZ has been shown to be dependent upon interaction with a conserved C-terminal region of ADC, deletion of which leads to loss of the protein-protein interaction[8]. Since this region is not conserved in the Bacillus ADC, we anticipated that PanZ would not be able to interact with this protein and, consistent with this hypothesis, overexpression of PanZ does not lead to β-alanine auxotrophy in an *E. coli ΔpanD::BspanD* strain (figure 2a). This provides the first direct evidence that it is the physical interaction between PanD and PanZ that leads to growth arrest in *E. coli* as result of PanZ overexpression.

The PanZ(N45A) mutant has previously been shown to be unable to complement a *panZ* deletion strain[12]. This is due to loss of a critical hydrogen-bond (Figure 1a) which reduces the affinity

between PanZ and proPanD from approximately 100 nM to 4 μM, such that, at physiological concentrations of PanD and PanZ, the complex does not form.[8] We therefore screened a range of site-directed mutants to identify any that could still complement the *panZ* deletion strain but were not susceptible to overexpression-induced growth inhibition. This screening process identified the site-directed mutants PanZ(R73A) and PanD(K119A) (figures 2b and 2c). In both cases, the mutated protein still complements the β-alanine auxotrophy of the *ΔpanZ* and *ΔpanD* strains respectively, indicating that catalytically active PanD is formed in both cases, but no growth inhibition is observed as a result of mutant PanZ overexpression.

The effect of both mutations on complex formation was assessed *in vitro* using size-exclusion chromatography of the purified proteins (see figures 2d and S3). While the wild-type PanD/PanZ complex elutes as a heterooctamer, the PanD(K119A)/PanZ complex eluted as a mixture of the heterooctamer complex and the individual proteins suggesting a weakened protein-protein interaction. No heterooctamer could be observed for the PanD/PanZ(R73A) system suggesting that these proteins do not form a stable complex. The fact that the PanZ(R73A) mutant can complement a *ΔpanZ* strain, which requires activation of PanD, suggests the proteins must be able to interact to some extent. Residue Arg73 is involved in AcCoA binding by PanZ – the δ-guanidino group forms a salt-bridge with Glu103, locking the pantetheine binding pocket (see figure S4). For this mutant, we therefore reinvestigated the protein-protein interaction using ITC and the inactivatable PanD(S25A) mutant[28]. For PanZ(R73A), we observed a 250-fold change in the affinity for AcCoA (220 ± 30 μM *c.f.* 0.8 μM for WT PanZ, figure 2d) but only a six-fold decrease in the affinity for PanD in the presence of excess AcCoA(figure 2e); global fitting yielded an estimate for this dissociation constant of 1.01 ± 0.27 μM, close to the previously determined affinity of 150 nM for WT PanZ for the S25A mutant[8]. The reduced affinity for

AcCoA is sufficient to account for the lack of observed complex by size-exclusion chromatography. Though the complex can form, it can only accumulate in the presence of high concentrations of AcCoA. This observation supports an AcCoA concentration-sensing role for PanZ; the reduced affinity for AcCoA means the inhibitory PanD-PanZ complex cannot accumulate at low concentrations of AcCoA.

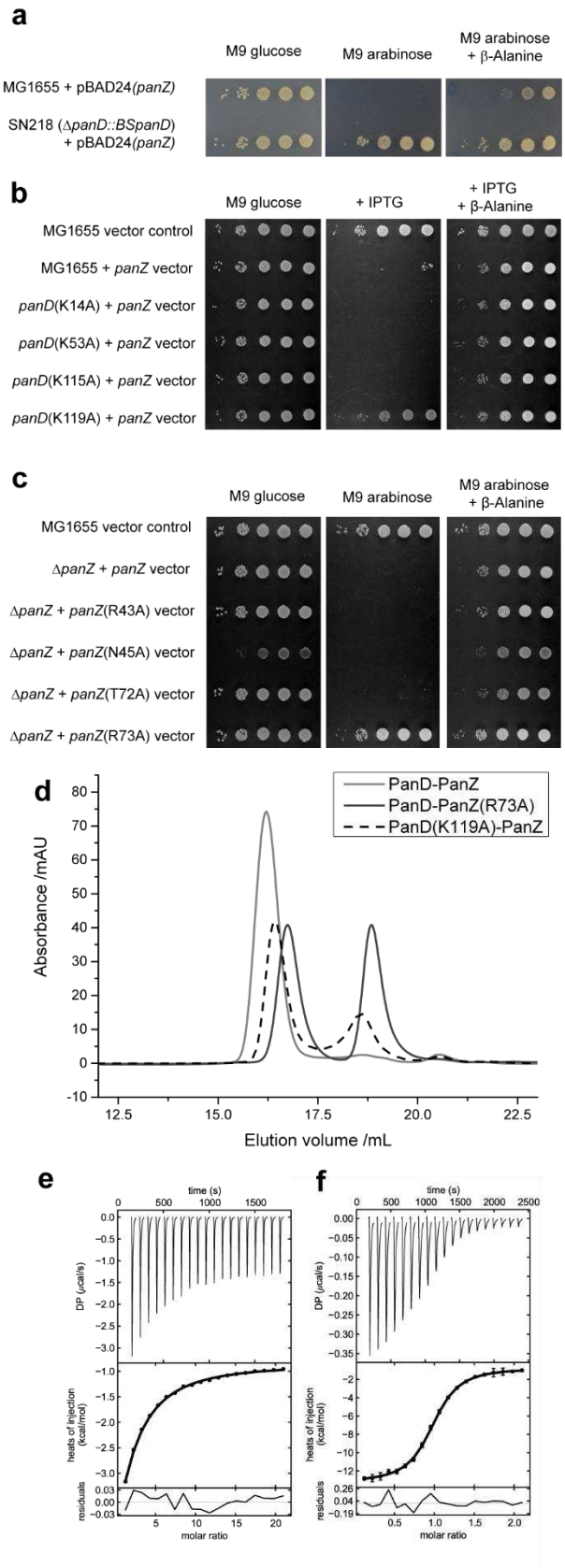

a
M9 glucose
M9 arabinose
M9 arabinose + β-Alanine
MG1655 + pBAD24(panZ)
SN218 (ΔpanD::BSpanD) + pBAD24(panZ)
b
M9 glucose
+ IPTG
+ IPTG + β-Alanine
MG1655 vector control
MG1655 + panZ vector
panD(K14A) + panZ vector
panD(K53A) + panZ vector
panD(K115A) + panZ vector
panD(K119A) + panZ vector
c
M9 glucose
M9 arabinose
M9 arabinose + β-Alanine
MG1655 vector control
ΔpanZ + panZ vector
ΔpanZ + panZ(R43A) vector
ΔpanZ + panZ(N45A) vector
ΔpanZ + panZ(T72A) vector
ΔpanZ + panZ(R73A) vector
d
PanD-PanZ
PanD-PanZ(R73A)
PanD(K119A)-PanZ
Absorbance /mAU
Elution volume /mL
e
f
time (s)
DP (μcal/s)
heats of injection (kcal/mol)
residuals
molar ratio

Figure 2 Regulation of ADC is due to CoA-dependent interaction of PanZ and ADC. a Overexpression of PanZ is sufficient to generate β-alanine auxotrophic bacteria. Growth of MG1655 is inhibited on M9 arabinose media in the absence of β-alanine. In contrast, growth of strain SN218, in which the *panD* locus is replaced with that from *B. subtilis,* is not perturbed by PanZ overexpression. **b** & **c** Screening of mutations in panZ and panD to identify site-directed mutants able to release inhibition but maintain growth. **b** A K119A mutation in the chromosomal *panD* locus leads to loss of growth suppression. **c** Overexpression of panZ(R73A) does not inhibit cell growth. **d** Analysis of protein complex formation by size-exclusion chromatography. The WT PanD-PanZ complex elutes as a heterooctamer (light grey line) whereas the PanD(K119A)-PanZ complex elutes as a mixture of the heterooctamer, PanD tetramer and PanZ monomer (dashed line). The PanD.PanZ(R73A) mixture elutes as independent tetramer and monomer components (dark grey line). **e** & **f** calorimetric analysis of interaction of PanZ(R73A) and AcCoA. **e** The loss of Arg73 from the AcCoA binding site reduces the affinity of the protein for AcCoA by ~250-fold. **f** Titration of PanZ(R73A) into PanD(S25A) in the presence of high concentrations of AcCoA (1 mM) indicates that the proteins interact at physiological concentrations of AcCoA[29].

High-potency growth inhibition by pentyl pantothenamide is dependent upon the PanD/PanZ interaction Inhibition of growth as a result of overexpression may not be physiologically relevant as if the cellular concentration of PanZ is significantly lower than that of PanD then the inhibited complex may not accumulate. Both proteins have been detected via high-throughput proteomic abundance screening of *E. coli* and these results suggests that the concentration of PanZ is slightly lower than that of PanD[30]. It is therefore possible that insufficient PanZ.CoA complex can form *in vivo* to markedly inhibit PanD. We therefore wished

to use a small molecule probe to investigate the behaviour of the proteins at natural concentrations. Pentyl pantothenamide (N5-Pan) has previously been shown to exhibit enhanced activity against *E. coli* in comparison to other bacteria, as discussed above[1-3,7]. Since N5-Pan has been shown to be metabolized to ethyl dethiacoenzyme A (EtdtCoA) and down-regulate CoA biosynthesis, we hypothesized that this effect was due to interaction of EtdtCoA with PanZ resulting in subsequent inhibition of PanD. PanZ binds CoA and AcCoA with equal affinity, suggesting that PanZ will also bind EtdtCoA since PanZ is insensitive to modification of its ligand in this region. We therefore generated EtdtCoA by a combination of chemical synthesis to form N-pentyl phosphopantothenamide followed by enzymatic turnoverusing CoaD and CoaE to form the target molecule. We then used ITC to investigate binding of EtdtCoA to WT PanZ (figure 3a). We observed tight binding, essentially indistinguishable from the signal observed for binding of AcCoA (K_d = 524 ± 64 nM) to the same protein sample.

If EtdtCoA inhibits cell growth by binding of the PanZ.EtdtCoA complex to ADC, a strain of *E. coli* in which PanZ and PanD cannot interact will be resistant, *i.e.* an *E. coli ΔpanD::BspanD* strain (which does not exhibit PanZ-mediated repression) should be resistant to pentyl pantothenamide. We therefore tested activity of N5-Pan against both *E. coli* MG1655 and the *E. coli* SN218 strain. As expected, for MG1655 we observed an MIC of ~2 μg/mL which increased to 50 μg/mL with β-alanine supplementation (figure 3b). For SN218, we observed an MIC of greater than 50 μg/mL, in the presence of β-alanine this increased to 100 μg/mL. Next, we investigated the inhibition of cell growth in liquid culture as a function of N5-Pan concentration (see figure 3d & e and figure S6). We observed a similar pattern of inhibition to that observed on solid media, with inhibition of growth apparent at 4 μg/mL for the WT strain. In contrast, 200 μg/mL N5-Pan is required for full growth inhibition of the *ΔpanD::BspanD* strain, with a similar

profile to that seen for WT *E. coli* (MG1655) in the presence of β-alanine (see figure S7a). This is fully consistent with N5-Pan acting by limiting the rate of pantothenate production - since the strain containing the Bacillus PanD is unregulated by PanZ it is likely that this strain has a higher level of pantothenate production, enabling growth at the higher concentrations of N5-Pan.

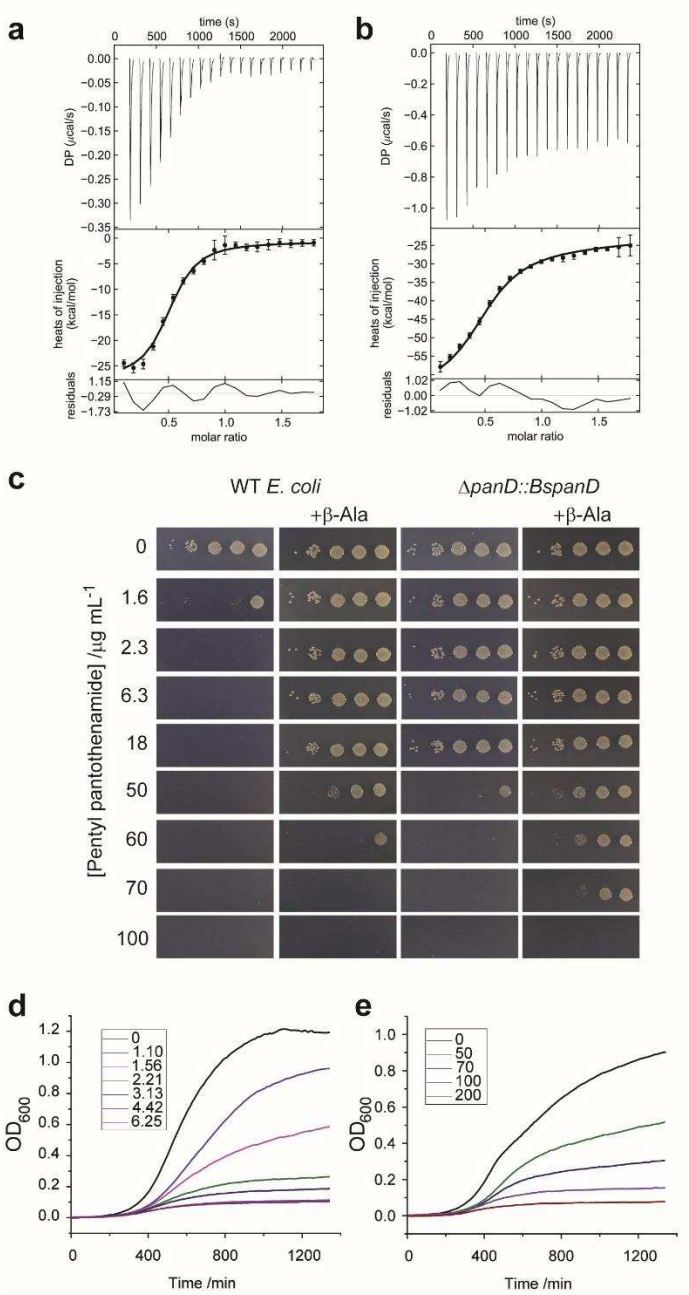


Figure 3 Testing of *E. coli* strains for growth inhibition by pentyl pantothenamide. a Titration of AcCoA against purified PanZ by ITC reveals sub-stoichiometric but tight binding of AcCoA, due to co-purification of CoA with PanZ[14]. **b** EtdtCoA binding to PanZ is indistinguishable from AcCoA (note sloping baseline due to residual salt in metabolite preparation, see supplementary figure S5 for details of global fitting). **c** Growth of WT *E. coli* (MG1655) and a *ΔpanD::BspanD* strain (SN218) on solid M9 agar medium supplemented with pentyl pantothenamide and β-alanine (0.5 mM). **d** & **e** Growth curves for MG1655 (d) & SN218 (*ΔpanD::BspanD*) (e) in liquid culture. Residual growth is observed even at inhibitory concentrations of compound.

In all cases, no change in the rate of cell growth is observed as a function of N5-Pan; instead, addition of N5-Pan restricts the final cell density at stationary phase. Even at inhibitory levels of compound, a small amount of residual growth is observed in direct proportion to the seeding density (see figure S7 b & c). If N5-Pan is inhibiting pantothenate biosynthesis then this corresponds to growth on residual available pantothenate in the cells, indicating that the cell normally maintains a cellular pool of pantothenate and coenzyme A many times the minimal amount required for growth. Intriguingly, the maximal growth level also varies between the strains – lower overall growth density is seen as a result of both β-alanine supplementation and substitution of the PanD (figures S6 and S7a) – this suggests that effective regulation of the coenzyme A biosynthetic pathway is critical to optimal cell growth.

DISCUSSION

In this work, we set out to confirm our original hypothesis, that binding of a PanZ.AcCoA complex to PanD regulates pantothenate biosynthesis *in vivo*[8]. Almost all vitamin biosynthetic

pathways in *E. coli* and other bacteria are tightly regulated and numerous mechanisms for this regulation have been identified over the last 40-50 years. These include feedback allosteric regulation activity of pantothenate kinase activity in the pathway from pantothenate to coenzyme A[31], DNA-binding transcription factors such as NadR which controls NAD biosynthesis[32], and metabolite-binding riboswitches which control numerous pathways including those for thiamine and cobalamin[33]. The consensus for the pantothenate biosynthetic pathway was that it is not regulated[34], however we recently proposed that the production of β-alanine is feedback regulated by the PanZ.AcCoA complex[8]. Our evidence for regulation was based on the overexpression phenotype of *panZ* and the *in vitro* inhibition of catalysis. To show that this inhibition is physiologically relevant we needed to demonstrate both that growth inhibition was due to the CoA-dependent PanD-PanZ interaction and that the inhibition occurred at native concentrations of PanD and PanZ in the cell. The first point is demonstrated here by two observations. Firstly, that it is the PanD-PanZ interaction that is required for the overexpression phenotype; substitution of the *E. coli panD* for the non-interacting Bacillus *panD* suppresses the phenotype. And secondly, that a site-directed mutant of PanZ with reduced affinity for CoA no longer elicits the overexpression phenotype, indicating that CoA binding by PanZ is required for inhibition. Demonstration of inhibition in the absence of overexpression was achieved using the compound N5-Pan[1]; its rapid metabolism to form EtdtCoA in the cell[2] provides a pool of a ligand for PanZ which would engage this regulatory pathway. The observation that *ΔpanD::BspanD* cells are resistant to the compound therefore supports our hypotheses that the PanD-PanZ complex is a both target for the metabolites of N5-Pan and that the complex can act in a regulatory fashion *in vivo*.

The crystal structure of the PanD-PanZ complex unexpectedly revealed a methyl ketone hydrate at the active site. This provides an additional mechanism for inhibition of catalytic activity. Even if the substrate, aspartate, were to bind non-covalently to the complex it cannot directly react with this form of the cofactor to produce the Schiff-base conjugate required for catalysis. This therefore provides an additional, kinetic barrier to substrate binding, allowing more effective inhibition even in the presence of the 4 mM aspartate[29] found in exponentially growing *E. coli*. (~50K_m)[10a]. We have previously hypothesized that PanZ was originally recruited to regulate catalytic activity during the evolution of the γ-proteobacteria[8], many of these organisms are commensal organisms which grow in nutritionally-rich environments in which pantothenate biosynthesis is dispensible. We suggest that a point mutation (or mutations) in the target protein, PanD, which enhance the affinity of the regulatory interaction may have inadvertently led to an absolute requirement for the interaction which leads to conversion of proPanD to PanD. This remains speculation, however, since no organism containing PanZ which does not also require it for activation has been identified[35] as yet.

The final finding in this work, that the enhanced activity of pentyl pantothenamide against *E. coli* is due to binding to PanZ, creates an opportunity to target this regulatory mechanism for antibiotic chemotherapy. What is the origin of this specificity? A vast array of pantothenamides have been synthesized and tested yet none have matched the potency of N5-Pan for inhibition of E. coli growth.[4,36]. The binding of PanZ to AcCoA, CoA and EtdtCoA are essentially indistinguishable and the structure of the acetyl binding pocket supports this observation; no specific hydrogen-bonding interactions occur to the acetyl group of AcCoA suggesting that loss of the carbonyl group will not lead to loss of binding affinity. The methyl of the acetyl moiety binds in a shallow pocket which can therefore readily accommodate the terminal methyl group of

EtdtCoA. However it may not be able to accommodate longer analogues, suggesting a rationale for the loss of activity in the series going from N5-Pan to N-heptyl pantothenamide. Since CoA binds with equal potency it appears that this binding pocket has little effect on the binding and we would therefore have expected shorter homologues e.g. methyldethiacoenzyme A or dethiacoenzyme A to bind equally well. Given that the antimetabolites N-butyl pantothenamide and N-propyl pantothenamide are less active, despite being substrates for *E. coli* PanK[4], suggests either that binding to PanZ is compromised or their metabolism in the cell by CoaD and CoaE is less effective. In any case, our structural model provides an opportunity for rational design and experimentation to identify new more potent inhibitors of this biosynthetic pathway.

CONCLUSION

In conclusion, we have demonstrated that growth inhibition as a result of PanZ overexpression is linked to physiologically-relevant regulation by the PanDZ complex of the pantothenate biosynthetic pathway in a cellular context. This demonstration was dependent upon the identification of the PanDZ complex as a target for the antimetabolite precursor pentyl pantothenamide (N5-Pan), providing the opportunity for structure-led design of novel compounds against this biosynthetic pathway.

ASSOCIATED CONTENT

Supplementary figures, further details of ITC data analysis, size exclusion chromatograph, full protocols for small molecule synthesis and associated analytical data can be found in supplementary information.

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Author Contributions

MEW, SN and HN conceived the project; SN constructed mutant strains, conducted growth assays and size-exclusion analysis; ZLPA synthesized pentyl pantothenamide and ethyl dethiacoenzyme A, purified proteins and conducted ITC analysis; HEM synthesized ethyl dethiacoenzyme A and conducted initial ITC analysis; DCFM and ARP carried out structural analysis of the WT PanD/PanZ complex; MEW analysed biophysical data, conducted growth assays and wrote the paper. All authors contributed to revision of the manuscript. SN and ZLPA contributed equally.

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ABBREVIATIONS

EtdtCoA: ethyldethiacoenzyme A, N5-Pan: N-pentyl pantothenamide

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