Article:
Morrison, PM, Balmforth, MR, Ness, SW et al. (4 more authors) (2016) Confirmation of a protein-protein interaction in the pantothenate biosynthetic pathway using Sortase-mediated labelling. ChemBioChem, 17 (8). pp. 753-758. ISSN 1439-4227

https://doi.org/10.1002/cbic.201500547

Confirmation of a protein-protein interaction in the pantothenate biosynthetic pathway using Sortase-mediated labelling

Philip M. Morrison,[a] Matthew R. Balmforth,[a] Samuel W. Ness,[a]† Daniel J. Williamson,[a] Michael D. Rugen,[a]‡ W. Bruce Turnbull, [a] and Michael E. Webb*[a]

School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom
E-mail: m.e.webb@leeds.ac.uk

† Now at: Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge
‡ Now at: John Innes Centre, Norwich Research Park, Norwich, United Kingdom

Abstract: High-throughput studies have been widely used to identify protein-protein interactions however the veracity of few of these candidate interactions have been demonstrated *in vitro*. We use a combination of isothermal titration calorimetry and fluorescence anisotropy to screen candidate interactions within the pantothenate biosynthetic pathway. In particular, we observe no interaction between the subsequent enzyme in the pathway, pantothenate synthetase (PS) and aspartate decarboxylase but do observe interaction of PS and the putative Nudix hydrolase, YfcD. Confirmation of the interaction by fluorescence anisotropy was dependent upon labelling of an adventitiously formed glycine on the protein N-terminal affinity purification tag using Sortase. Subsequent formation of the protein-protein complex led to apparent restriction of the dynamics of this tag, suggesting that this approach could be generally applied to a subset of other protein-protein interaction complexes.

Introduction

Pantothenate (vitamin B_5) is an essential metabolite for all organisms and forms part of coenzyme A (CoA) and the phosphopantetheine moiety of acyl carrier proteins[1]. However, it is only synthesized in bacteria, plant and fungi and represents a potential herbicidal and antibiotic therapeutic target. The biosynthetic pathway to pantothenate consists of four essential steps (scheme 1), three of which are common to all organisms. α-Ketoisovalerate **1**, an intermediate in valine biosynthesis, is converted to D-pantoate **3** via the sequential action of ketopantoate hydroxymethyltransferase (KPHMT encoded by *panB*) and a reductase (ketopantoate reductase (KPR encoded by *panE*) or acetohydroxyacid isomeroreductase (AHIR encoded by *ilvC*) in *Escherichia coli*). Pantothenate is then formed via the ATP-dependent condensation of pantoate **3** with β-alanine **5** catalyzed by pantothenate synthetase (PS, encoded by *panC*). The source of β-alanine is organism-dependent; in bacteria, β-alanine is generated via the α-decarboxylation of aspartate **4** by aspartate α-decarboxylase (ADC, encoded by *panD*). Several of the enzymes in the pathway have been the subject of extensive drug discovery efforts including PS[2] and ADC[3].

We have recently identified a fifth essential protein for the biosynthetic pathway that is limited to a subset of γ-proteobacteria including *Escherichia coli*[4] and *Salmonella enterica*[5]*.* This protein, PanZ, is essential for the maturation of the expressed PanD zymogen to form the pyruvoyl cofactor found in mature ADC. Subsequent biophysical and biochemical characterization has shown that this interaction and activation is CoA-dependent[6]. This interaction was not observed in high-throughput proteomic screens for protein-protein interactions, although other candidate interactions had been identified using pull-down approaches. For example, Arifuzamann *et al.* used affinity copurification coupled with MALDI-MS to identify interactions between PS and a protein of unknown function YfcD[7] as well as between PS and ADC. The structure of YfcD has been solved as part of high-throughput structural genomics (PDB: 2fkb) and based on sequence and structural conservation, has been classified as a potential NUDIX hydrolase but no role in the pantothenate biosynthetic pathway had been identified. In this study, we wished to verify both of these candidate interactions using biophysical approaches to build a more complete picture of the biosynthetic pathway.

In order to conduct these studies we needed to generate fluorescently-labelled protein. Fluorescence labelling of proteins is commonly carried out using chemoselective but non-specific reagents such as maleimides and activated NHS-esters. Recently, the transpeptidase sortase A (SrtA) from *Staphylococcus aureus* has been used for the *in vitro* ligation of proteins[8] to a range of substrates including dyes, nucleic acids, and other proteins. This enzyme catalyses the transpeptidation of the C-terminal peptide sequence LPXTG by transfer of the LPXT to a reactive cysteine residue in the enzyme active site.[9] An N-terminal glycine in a protein or peptide can then intercept this thioester intermediate to generate a modified peptide or protein. While the native reaction is reversible, modifications of the LPXTG C-terminal

unit can allow irreversible ligation, improving the yield of *in vitro* labelling reactions and requiring fewer equivalents of label. Effective modifications include the use of a depsipeptide linkage in the C-terminal glycine[10] and the elimination of a diglycine-depsipeptide derivative which can then cyclise to form diketopiperazine.[11] Generally, however, production of proteins to be labelled has required cloning of new protein constructs to incorporate C-terminal LPXTG recognition sequences or N-terminal glycine residues. In this study, we report the use of SrtA to fluorescently label His-tagged PS using an adventitiously formed N-terminal glycine and the application of this protein in our biophysical characterization.

Results and Discussion

We initially overexpressed His-tagged PS and YfcD in *E. coli* C41 (DE3) using the pCA24N(-GFP) constructs described in the Arrifuzamann study[7]. These constructs encode N-terminally His-tagged proteins together with a small C-terminal extension left after removal of a C-terminal GFP tag. His-tagged ADC(T57V) and PanZ were overexpressed in *E. coli* Δ*panZ* Δ*panD* (DE3) as described previously[6a]. All proteins were purified by gel filtration and exhibited largely monodisperse chromatograms with the exception of YfcD which showed a variety of multimeric forms. For biophysical analysis of YfcD, the major peak corresponding to a monomeric species was used.

Following purification, interaction of PS with the three other proteins was initially assessed using ITC. Firstly, we were surprised to observe apparent dissociation of PS multimers upon dilution of the protein into buffer (see Figure 1a). This observation contradicted previous studies of the enzyme. The crystal structure of the enzyme[12] shows PS has a dimeric structure which could be consistent with the apparent behavior in solution, but no evidence for a monomer-dimer or dimer-multimer equilibrium has been reported previously. However, subtraction of this dilution experiment (Figure 1a) from a titration of PS into YfcD (Figure 1b) did provide a curve, albeit a rather featureless one (Figure 1c), and while this cannot be used to obtain robust estimates for binding constants it did suggest that the putative interaction between YfcD and PS does occur. The corresponding titration of YfcD into PS yielded a low affinity titration curve (See supplementary figure 1) suggesting an affinity of approximately 30 μM, however it was not possible to obtain a robust estimate for the stoichiometry of interaction from this experiment. In contrast, titration of ADC into PS and PS into ADC (using the inactivatable T57V mutant[13], see supplementary figure 2) revealed essentially no interaction suggesting that the MS-identified interaction of ADC and PS does not occur. This was consistent with our earlier studies of PS as a candidate activating factor for ADC; partially-purified, untagged PS was incubated with purified WT ADC but no enhancement in rate of PanD maturation was observed (data not shown).

Closer inspection of the protein expression constructs used by Arifuzamann *et al.* to generate His-tagged PS and YfcD revealed that a residual cloning scar had introduced a additional C-terminal cysteine residue[14]. The *E. coli* ORFeome library was originally constructed by cloning into pCA24N to generate a set of N-terminally His-tagged, C-terminally GFP-tagged proteins before removal of the GFP tags to generate the library in the pCA24N(-GFP) background leaving the cloning scar.[14] We hypothesised that the C-terminal extension leads to the observed apparent dissociation behaviour and therefore subcloned the PS-coding sequence into pET28a. PS expressed using this vector no longer aggregates and dilution into buffer now gave a flat trace corresponding to dilution rather than dissociation (Figure 1d). Using this construct it was possible to confirm by ITC that PS and YfcD interact with low micromolar affinity (2 μM) in a 2:1 ratio (Figure 1e).

We next sought to use sortase-mediated fluorescence labelling to independently confirm the occurrence of the interaction. Here, the use of the pET28a vector proved advantageous as the resulting affinity tag starts with MGSS and the N-terminal methionine is therefore cleaved *in vivo* by methionine aminopeptidase to leave an accessible N-terminal glycine residue in the expressed protein.[15] (NB In our previous report of quantitative labelling of N-terminal glycine residues using depsipeptide substrates,[10, 16] the sortase enzyme was also expressed from pET28a and was thus fluorescently tagged along with the intended substrate protein.) In those experiments, it proved convenient to remove the sortase by Ni-NTA chromatography after the labeling reaction was complete. However, in the current study, both the sortase and PS proteins were N-terminally His-tagged precluding their rapid separation by affinity purification. The SrtA gene was therefore subcloned into the pMalc5x vector to create constructs encoding either MBP-SrtA or MBP-His-SrtA conjugates. Both proteins overexpressed well, however the maltose used to elute the MBP-tag during amylose-affinity chromatography is not readily removed by dialysis, which led to reduced efficiency of MBP-SrtA on amylose resin after the ligation reaction. The MBP-His-SrtA construct was therefore used preferentially in subsequent experiments. This protein was purified to homogeneity using sequential Ni-NTA affinity chromatography and size-exclusion chromatography to avoid the use of maltose.

Under our standard conditions for labelling proteins using His-tagged SrtA and a FITC-conjugated depsipeptide **7** (2.5 equivalents peptide, 10 mol% SrtA, 4 hours), the MBP-His-SrtA construct proved less active than His-SrtA (see supplementary figure 3). Nevertheless, the PS protein was successfully labelled with the fluorophore (figure 2). The product was then purified by sequential separation by amylose-affinity (to remove MBP-His-SrtA) and Ni-NTA affinity (to remove free dye). Unfortunately, following elution of the fluorescent PS with imidazole, it was found that trace amounts of MBP-His-SrtA remained in the product. As an excess of the amylose resin had been used during the purification process, we posited that a small portion of MBP-His-SrtA may have become bound to maltose in the bacterial growth

media thus precluding its interaction with the amylose resin. For biophysical analysis using fluorescence anisotropy the protein was therefore further purified by size-exclusion chromatography. Usually a relatively small fluorescent ligand is required for fluorescence polarization and anisotropy experiments so that the fluorescence may show an increase in anisotropy upon binding to a large receptor protein. For a labelled molecule the size of PS (~60 kDa dimer), one would expect to observe a fluorescence anisotropy value that is already close to the theoretical maximum ($r_0 = 0.4$), therefore, such systems would not normally be suitable for anisotropy experiments. However, the flexibility of the N-terminal His-tag allows significant local motions that result in the observation of an intermediate value for the anisotropy (~0.15). Titration of YfcD into the labelled PS sample led to a small decrease in the fluorescence intensity (see supplementary figure 4) but a proportionally larger, concomitant increase in the fluorescence anisotropy (figure 3). These changes are consistent with an interaction between YfcD and PS that restricts the motion of the tag on PS, while possibly forming a non-specific interaction of the fluorescein label with YfcD.

Curve fitting (following correction for the observed fluorescence quenching) revealed an apparent affinity of the interaction (figure 3b, $K_{0.5}$ 2.16 ± 0.11 μM), consistent with the estimated affinity from calorimetry (2.11 ± 0.46 μM). To confirm that the interaction was not due to binding of YfcD with the labelled portion of the protein, unlabelled PS was titrated into a mixture of the FITC-labelled PS and YfcD to give an observed IC_{50} of 16.9 ± 0.28 μM (supplementary figure 5). Fitting of the data for both experiments could only be achieved using a cooperative binding model (Hill coefficient ~2). Given that ITC suggests a 1:2 ratio between PS and YfcD monomers and PS exists as a dimer in solution, such cooperativity suggests formation of a large multisubunit complex between the proteins. Given the experimentally estimated abundances for both proteins in the *E. coli* cell[17] which correspond to approximately 360 nM for YfcD and 900 nM for PS[18], the affinity of this interaction suggests that significant quantities of a protein-protein complex can form *in vivo* and that though the majority of the protein will not be in such a complex, its formation is physiologically relevant. Ultimately, assessment of the significance of the complex requires further work, in particular elucidation of the catalytic function of YfcD and the effect of the protein-protein interaction on PS and YfcD catalytic activity.

Conclusions

In conclusion, we have demonstrated that the putative PS-YfcD interaction identified by high-throughput approaches can be reconstituted *in vitro*. We have used MBP-tagged sortase to mediate N-terminal labelling of pantothenate synthetase. This protocol is readily extendable to other N-terminally His-tagged proteins with an N-terminal glycine. The labelled PS was used in a fluorescence anisotropy assay to validate complexation of PS and the uncharacterised protein YfcD previously observed by ITC. We propose that the success of this experiment is principally due to interaction of the proteins in the region of the N-terminal tag and that this approach may be generalizable to other systems. While high-throughput studies using affinity purification linked to mass spectrometry and yeast two-hybrid assays have generated databases with large numbers of potential protein-protein interactions, these often give no indication of the likely affinity and therefore physiological relevance of the interactions. The labelling approach and accompanying biophysical method (fluorescence anisotropy) we have used is readily adaptable to parallel characterisation of that subset of protein-protein interactions which occur near the N-terminus of one or both partner proteins and therefore has potential for the high-throughput approaches to protein-protein interactions.

Experimental Section

Overexpression and purification of PS from pCA24N(-GFP) His-tagged pantothenate synthetase with a C-terminal extension (PS-[LCGR}) was overexpressed and purified using standard immobilised metal affinity chromatography. *E. coli* BL21 (DE3) were transformed with pCA24N (-GFP) plasmid by electroporation. Protein was overexpressed using autoinduction for 18 hours at 37 °C and cells isolated by centrifugation (10000*g*, 15 min). Cells were resuspended in 50 mM Tris pH 7.4, 300 mM NaCl, 10 mM imidazole and lysed by passing through a cell disrupter (20 kpsi). The lysate was cleared by centrifugation (30000 *g*, 45 min) and after addition of DNase I, the cleared lysate was applied to an equilibrated column of Ni-NTA agarose (Qiagen). The column was washed with lysis buffer and lysis buffer supplemented with 50 mM imidazole before elution of protein with lysis buffer containing 250 mM imidazole. Protein-containing fractions were identified by SDS-PAGE and concentrated to total volume of 1 mL by centrifugal concentration (Vivaspin, 10k MWCO). The protein was applied to a Superdex 75 26/60 size-exclusion column and isocratically eluted in 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl.

Overexpression/purification of PS from pET28a PS-coding DNA was amplified from the pCA24N(-GFP) vector using Pwo DNA polymerase and primers PSBamH1 and NheIPS. Following PCR, the template strand was digested with DpnI (1 μl) for 1 h at 37 °C. PCR product was purified using a modified QIAquick® Gel extraction protocol omitting gel purification steps. Purified product and pET28a vector were digested using BamHI-HF and NheI-HF for 1 h at 37 °C and purified following the modified QIAquick® Gel extraction protocol. Ligation was undertaken using T4 DNA ligase for 30 min before transformation into chemically-competent *E. coli*

XL10 cells followed by growth on LB-agar (Kan) plates overnight at 37 °C. Single colonies were grown in LB media overnight and the plasmid pET28A-PS isolated via alkaline lysis (Qiagen miniprep) and the sequence confirmed by DNA sequencing (GATC Biotech). (PSBamHI: ATGAGGATCCTTACGCCAGCTCGACCAT, NheIPS: ATTGGCTAGCATGTTAATTATCGAAACCCTGCCGC)

His-tagged pantothenate synthetase (PS) was overexpressed and purified using standard immobilised metal affinity chromatography. *E. coli* C41 (DE3) were transformed with the plasmid pET28a-PS using electroporation. Protein was overexpressed using autoinduction for 18 h at 37 °C and cells were isolated by centrifugation (10000 *g*, 15 min). Cells were resuspended, lysed and the protein purified by Ni-NTA and size exclusion chromatography as detailed for PS-pCA24N(-GFP) above.

Overexpression/purification of YfcD His-tagged YfcD was overexpressed and purified using standard immobilised metal affinity chromatography. *E. coli* C41 (DE3) cells were transformed with the plasmid pCA24N(-GFP)-YfcD by electroporation. Protein was overexpressed using autoinduction for 25 hours at 37 °C and cells isolated by centrifugation (10000 *g*, 15 min). Cells were resuspended, lysed and the protein purified by Ni-NTA and size exclusion chromatography as detailed for PS-pCA24N(-GFP) above.

Overexpression/purification of MBP-His-SrtA His-SrtA was cloned from pET28a into pMalC5X using NcoI and EcoRI restriction sites and the sequence of the plasmid confirmed by DNA sequencing. Protein was overexpressed using autoinduction in *E. coli* BL21-G (DE3) for 25 h and cells isolated by centrifugation (10000 g, 15 min). Cells were resuspended in 50 mM HEPES pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl and lysed by passing through a cell disrupter (25 kpsi). The lysate was cleared by centrifugation (35000*g*, 45 min) and filtered through a 0.8 μM filter. The cleared lysate was applied to an equilibrated column of Ni-NTA agarose (Qiagen). The column was washed with lysis buffer and lysis buffer supplemented with 50 mM imidazole before elution of protein with lysis buffer containing 500 mM imidazole. Protein-containing fractions were identified by SDS-PAGE and concentrated to total volume of 1 mL by centrifugal concentration (Vivaspin, 10k MWCO). The protein was applied to a Superdex 75 26/60 size-exclusion column and isocratically eluted in 50 mM HEPES pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl.

Labelling protocol PS, MBP-His-SrtA and **1** were combined and diluted with 50 mM HEPES pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl to final concentrations of 60 μM, 12 μM, and 120 μM respectively in a total reaction volume of 1 mL. Following the 4 hour incubation, the reaction mixture was passed down an amylose column and eluted with buffer A (50 mM Tris, 25 mM $MgCl_2$, 120 mM NaCl, pH 7.6, 15 mL). PS was isolated by passing elutions through a Ni-NTA column. The column was washed with buffer A supplemented with 50 mM imidazole (5 mL) before eluting with Buffer A supplemented with 500 mM imidazole (25 mL). PS-containing fractions were identified by SDS-PAGE and concentrated to total volume 1 mL by centrifugal concentration (Vivaspin, 10k MWCO). The protein was applied to a Superdex 75 16/60 size-exclusion column and isocratically eluted in 50 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl pH 7.4. Protein and FITC concentrations were determined using a Nanodrop 2000 spectrophotometer.

Isothermal titration calorimetry ITC experiments were undertaken at 25 °C using a Microcal VP-ITC calorimeter with data analysis using Origin and NITPIC.[19] Protein solutions were concentrated using Amicon spin filters (10 kDa molecular weight cut-off) and the concentration measured using the Beer-Lambert equation with the absorbance taken at 280 nm wavelength. Extinction coefficients were calculated using Expasy Protparam (PS: 13075 $M^{-1}\,cm^{-1}$, YfcD: 26470 $M^{-1}\,cm^{-1}$) The cell was loaded using a gas-tight syringe with either protein solution or buffer from the spin filter flow-through for binding and dilution titrations respectively. The syringe was loaded with protein solution and any excess removed by blotting before the syringe was loaded into the calorimeter. After equilibration at 25 °C, 29 injections of 10 μL were spaced at either 120 or 180 seconds. The first injection was discarded from fitting. Data was processed and analysed using Origin VP ITC software. To obtain the approximate binding curve of PS-pCA24N(-GFP) (PS-[LCGR]) with YfcD, the values of heat of dilution was subtracted from the observed binding isotherms to give an approximate binding curve.

Fluorescence anisotropy Fluorescence polarization assays were carried out in 96 well plates (160 μL per well). All experiments were performed in 50 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl pH 7.4. A 2.5-fold dilution series of YfcD (33 μM to 15 nM) was titrated against a fixed concentration of FITC-PS (120 nM; 20% labelled, total concentration of PS 600 nm) in triplicate. For the competition assay, a 2.5-fold dilution series of unlabelled PS (153 μM to 500 nM) was titrated against a fixed concentration of FITC-PS (120 nM, total PS concentration 600 nM) and YfcD (2.33 μM) in triplicate. Proteins were mixed in the plate wells (final volume 155 μL) before data acquisition using a Perkin Elmer EnVision 2103 MultiLabel plate reader (excitation 480 nm, emission 535 nm, 5nm bandwidth). Details of the analysis are included in the supplementary information.

Acknowledgements

PMM and MRB were supported by studentships from the Wellcome Trust (096687/Z/11/Z and 102576/Z/13/Z). D J. Williamson was supported by an EPSRC top-slice studentship and the BBSRC (BB/M005666/1). We acknowledge continuing support for equipment and facilities from EPSRC (EP/K03135X/1) and the Wellcome Trust (062164 and 094232/Z/10/Z)

Keywords Sortase labelling • Fluorescence anisotropy • Pantothenate biosynthesis

References

[1] M. E. Webb, A. G. Smith, C. Abell, Nat. Prod. Rep. 2004, 21, 695-721.

[2] a. A. W. Hung, H. L. Silvestre, S. Wen, A. Ciulli, T. L. Blundell, C. Abell, Angewandte Chemie 2009, 48, 8452-8456; b. A. Kumar, A. Casey, J. Odingo, E. A. Kesicki, G. Abrahams, M. Vieth, T. Masquelin, V. Mizrahi, P. A. Hipskind, D. R. Sherman, T. Parish, PloS one 2013, 8, e72786; c. F. Ntie-Kang, S. Kannan, K. Wichapong, L. C. Owono Owono, W. Sippl, E. Megnassan, Mol. Biosyst. 2014, 10, 223-239; d. G. Samala, P. B. Devi, R. Nallangi, P. Yogeeswari, D. Sriram, Eur. J. Med. Chem. 2013, 69, 356-364; e. H. L. Silvestre, T. L. Blundell, C. Abell, A. Ciulli, Proc. Natl Acad. Sci. U. S. A. 2013, 110, 12984-12989.

[3] a. J. de Villiers, L. Koekemoer, E. Strauss, Chem.-Eur. J. 2010, 16, 10030-10041; bM. E. Webb, E. Stephens, A. G. Smith, C. Abell, Chem. Commun. (Camb) 2003, 2416-2417.

[4] S. Nozaki, M. E. Webb, H. Niki, MicrobiologyOpen 2012, 1, 298-310.

[5] T. N. Stuecker, K. M. Hodge, J. C. Escalante-Semerena, Mol. Microbiol. 2012, 84, 608-619.

[6] a. D. C. Monteiro, M. D. Rugen, D. Shepherd, S. Nozaki, H. Niki, M. E. Webb, Biochem. Biophys. Res. Commun. 2012, 426, 350-355; b. D. C. F. Monteiro, V. Patel, C. P. Bartlett, S. Nozaki, T. D. Grant, J. A. Gowdy, G. S. Thompson, A. P. Kalverda, E. H. Snell, H. Niki, A. R. Pearson, M. E. Webb, Chem. Biol. 2015, 22, 492-503; c. T. N. Stuecker, A. C. Tucker, J. C. Escalante-Semerena, MBio 2012, 3.

[7] M. Arifuzzaman, M. Maeda, A. Itoh, K. Nishikata, C. Takita, R. Saito, T. Ara, K. Nakahigashi, H. C. Huang, A. Hirai, K. Tsuzuki, S. Nakamura, M. Altaf-Ul-Amin, T. Oshima, T. Baba, N. Yamamoto, T. Kawamura, T. Ioka-Nakamichi, M. Kitagawa, M. Tomita, S. Kanaya, C. Wada, H. Mori, Genome Res. 2006, 16, 686-691.

[8] a. M. W. Popp, J. M. Antos, G. M. Grotenbreg, E. Spooner, H. L. Ploegh, Nat. Chem. Biol. 2007, 3, 707-708; b. H. Mao, S. A. Hart, A. Schink, B. A. Pollok, J. Amer. Chem. Soc. 2004, 126, 2670-2671.

[9] M. W. Popp, H. L. Ploegh, Angewandte Chemie 2011, 50, 5024-5032.

[10] D. J. Williamson, M. A. Fascione, M. E. Webb, W. B. Turnbull, Angew. Chem. Int. Ed. 2012, 51, 9377-9380.

[11] F. Liu, E. Y. Luo, D. B. Flora, A. R. Mezo, J. Org. Chem. 2014, 79, 487-492.

[12] a. F. von Delft, A. Lewendon, V. Dhanaraj, T. L. Blundell, C. Abell, A. G. Smith, Structure 2001, 9, 439-450; b. S. Wang, D. Eisenberg, Protein Sci. 2003, 12, 1097-1108.

[13] M. E. Webb, B. A. Yorke, T. Kershaw, S. Lovelock, C. M. C. Lobley, M. L. Kilkenny, A. G. Smith, T. L. Blundell, A. R. Pearson, C. Abell, Acta Cryst. 2014, D70, 1166-1172.

[14] M. Kitagawa, T. Ara, M. Arifuzzaman, T. Ioka-Nakamichi, E. Inamoto, H. Toyonaga, H. Mori, DNA Res. 2005, 12, 291-299.

[15] T. Meinnel, Y. Mechulam, S. Blanquet, Biochimie 1993, 75, 1061-1075.

[16] D. J. Williamson, M. E. Webb, W. B. Turnbull, Nat. Protoc. 2014, 9, 253-262.

[17] M. Wang, M. Weiss, M. Simonovic, G. Haertinger, S. P. Schrimpf, M. O. Hengartner, C. von Mering, Mol. Cell. Proteomics 2012, 11, 492-500.

[18] These concentrations are converted from relative abundance figures using an assumed volume of 0.6 mm3 with approximately 2.5 x 106 proteins/cell

[19] S. Keller, C. Vargas, H. Zhao, G. Piszczek, C. A. Brautigam, P. Schuck, Anal. Chem. 2012, 84, 5066-5073.

Figure Captions

Scheme 1. Pathway to pantothenate in *E, coli* and schematic of characterized and predicted and confirmed protein-protein interactions in the pathway. α-Ketoisovalerate **1** is converted to pantoate **3** by the action of ketopantoate hydroxymethyltransferase (KPHMT) and either ketopantoate reductase (KPR) or acetohydroxyacid isomeroreductase (AHIR). Pantothenate synthetase (PS) catalyses the ATP-dependent ligation of β-alanine **5** and pantoate to form pantothenate. Interaction of PanZ with aspartate decarboxylase (ADC) is required both for activation of the PanD zymogen to form ADC and for subsequent regulation of ADC catalytic activity.

Figure 1. Calorimetric analysis of interaction between PS and YfcD. Removal of the C-terminal cloning scar eliminates dissociation artifact in PS titrations. (a) Characteristic trace corresponding to dissociation observed upon titration of PS(pCA24N) (514 μM) into buffer. (b) Titration of PS(pCA24N) (514 μM) into YfcD (82 μM). (c) Approximate binding curve obtained from subtracting the dilution titration (a) from the binding titration (d) Titration of PS(pET28a) (232 μM) into buffer (e) Interaction observed when PS(pET28a) (232 μM) is titrated into YfcD (23 μM) with relevant binding data shown in table below.

Figure 2. (a) The depsipeptide used in SrtA labelling reactions to conjugate FITC to the N-terminal glycine of PS. (b) Coomassie blue stained 12% SDS PAGE gel demonstrating the labelling and purification process. In a 1 mL reaction volume, PS (60 μm) was labelled using MBP-His-SrtA (12 μm) and depsipeptide 1 (120 μm). MBP-His-SrtA and associated fragments were removed by passing through an amylose column and the PS isolated by Ni-NTA agarose affinity purification. Lane 1, protein marker; lane 2, crude labelling reaction mixture; lane 3, flow-through (FT) fraction from anylose column; lanes 4-6 fractions from Ni-NTA column: 3 flow-through, 5, 50 mM imidazole wash, lane 6, 250 mM imidazole elution The UV fluorescence (302 nm excitation range 520-640 nm) stationimage of lanes 2, 3 and 6 shows the presence of FITC-conjugated species in these lanes.

Figure 3. Fluorescence anisotropy measurements of interaction between PS and YfcD. (A) Titration of YfcD into 580 nM PS (120 nM labelled with FITC). (B) Fraction-bound values were fitted with the Hill equation to obtain $K_{0.5}$ = 1.87 ± 0.08 μM and Hill coefficient of 2.09 ±0.14 suggesting strongly cooperative binding between PS and YfcD. The fit to the Hill equation (blue) was significantly better ($p<0.001$) than a single-site binding model (orange).

Scheme 1

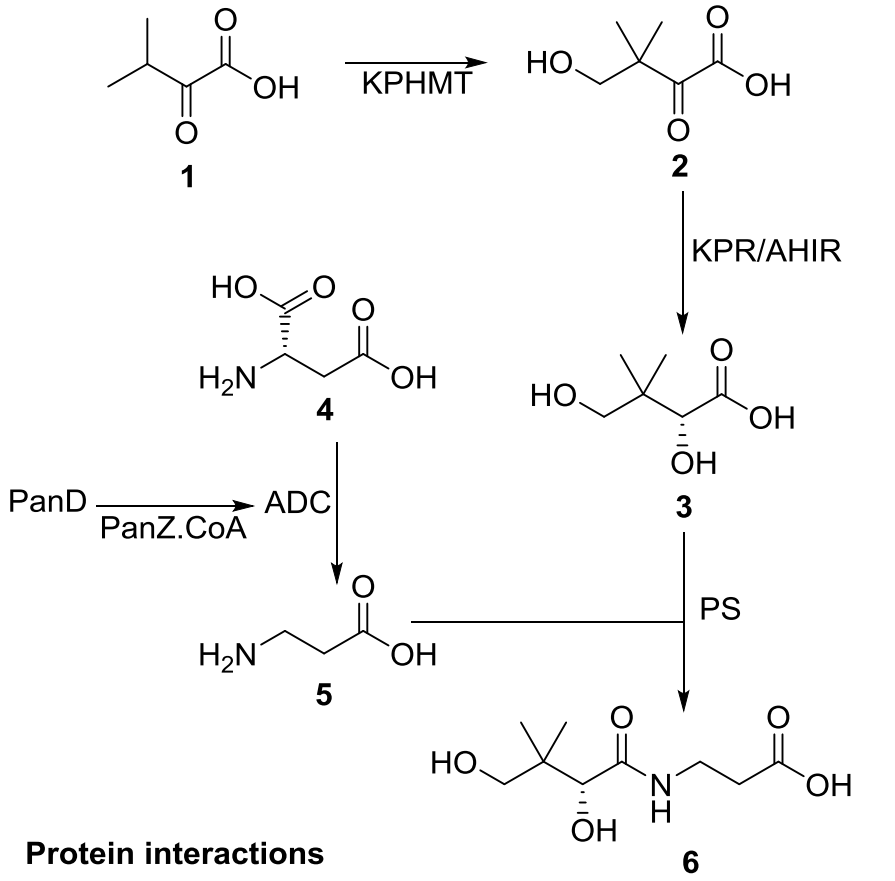

KPHMT
KPR/AHIR
PanD
PanZ.CoA
ADC
PS
1
2
3
4
5
6
Protein interactions
YfcD
PS
ADC
PanZ
predicted
confirmed

Figure 1

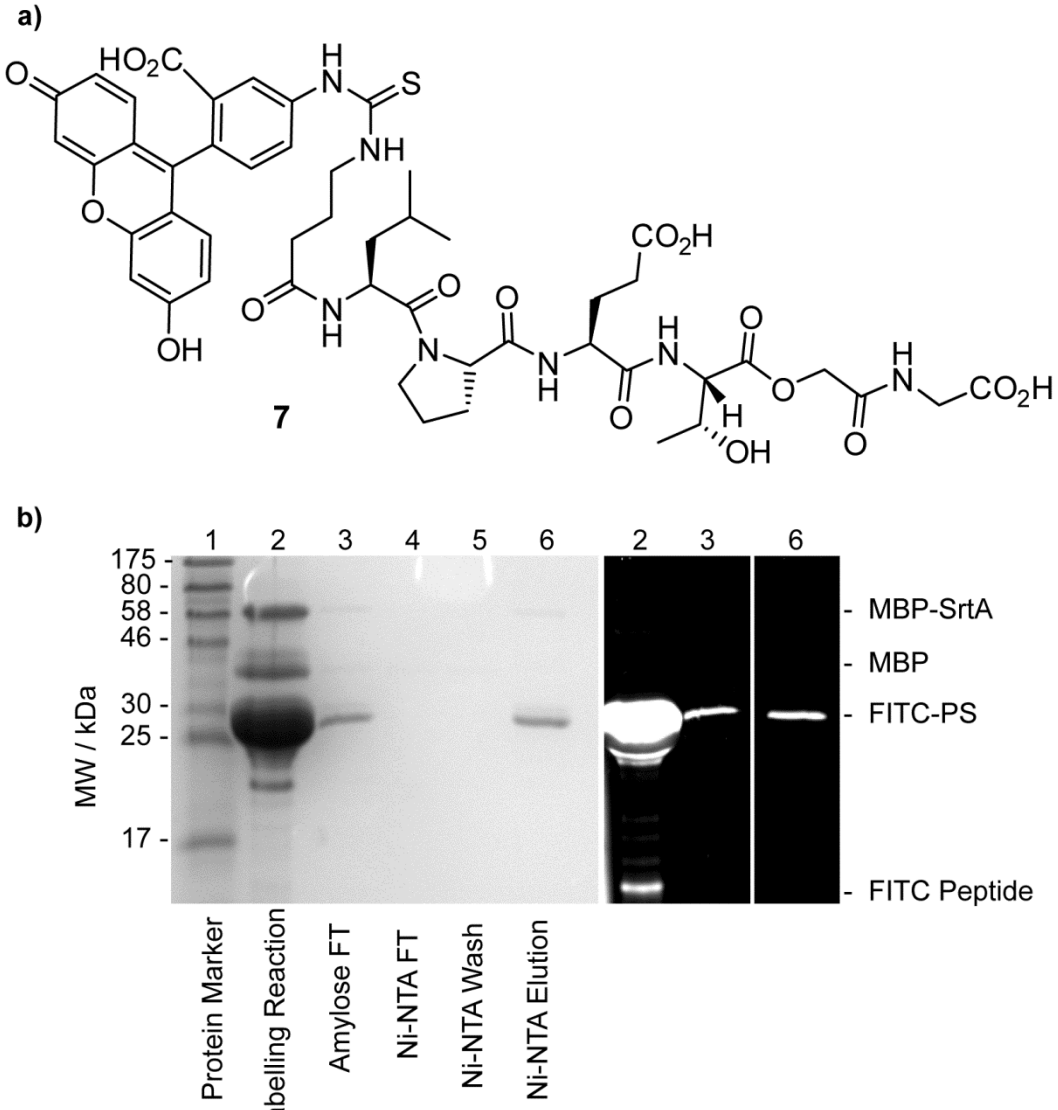
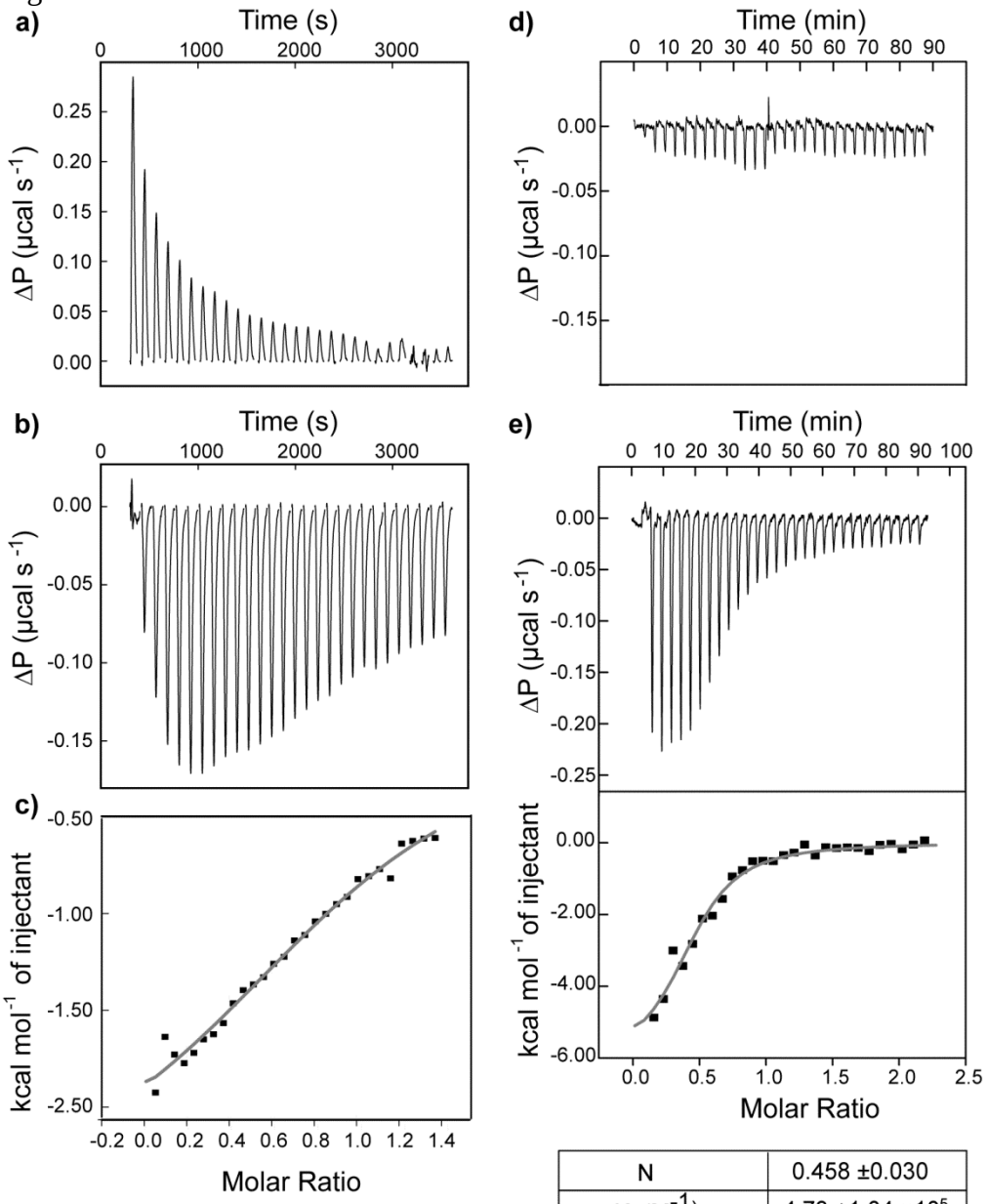
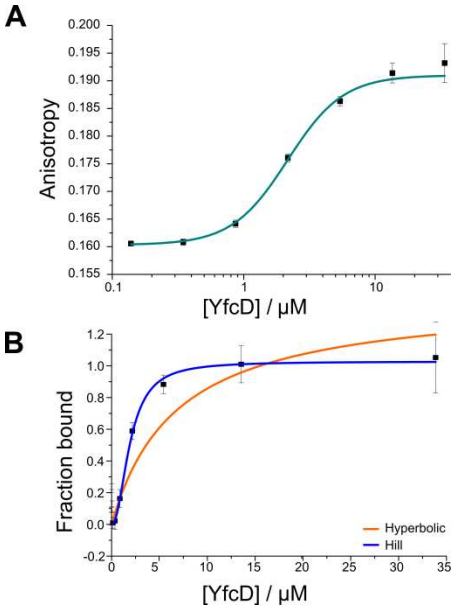

a)
7
b)
1 2 3 4 5 6 2 3 6
175
80
58
46
30
25
17
MW / kDa
MBP-SrtA
MBP
FITC-PS
FITC Peptide
Protein Marker
Labelling Reaction
Amylose FT
Ni-NTA FT
Ni-NTA Wash
Ni-NTA Elution

Figure 2



N	0.458 ±0.030
$K_a(M^{-1})$	4.73 ±1.04 ×10^5
ΔH (cal mol^{-1})	-6140 ± 550.4

Figure 3


A
Anisotropy
0.200
0.195
0.190
0.185
0.180
0.175
0.170
0.165
0.160
0.155
0.1
1
10
[YfcD] / µM
B
Fraction bound
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
5
10
15
20
25
30
35
Hyperbolic
Hill
[YfcD] / µM