

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Instruments

2.1.1.1 Protein Production, Purification and Mutagenesis

Cell lines were transformed using a **MiniPulser electroporation system** (BioRad), and growth plates were incubated in a **WTC incubator** (Binder). Cell lines were grown in a **Series 25 orbital incubator** (New Brunswick), and optical densities of cell cultures were determined using a **UV 1101 Biotech photometer** (WPA). Cell culture centrifugations were carried out in an **Evolution RC centrifuge** (Sorvall), using either a SLC-3000 or SS-34 rotor. Protein concentration centrifugations were conducted in a **CS-15R centrifuge** (Beckman). Cell lysis was performed on a **Soniprep 150 sonicator** (Sanyo), and proteins were purified using a **HiTrap® Chelating Column** (GE Healthcare) attached to an **ÄKTA Prime** (Amersham Pharmacia Biotech). Protein samples were analysed by SDS-PAGE using a **Mini-Protean protein electrophoresis system** (BioRad), and gel images were taken in a **G:Box gel imager** (Syngene). Polymerase chain reactions were conducted in a **Techgene thermocycler** (Techne), with DNA and protein concentrations determined by UV using a **Nanodrop800** (Thermo Scientific).

2.1.1.2 Mass Spectrometry

Waters SYNAPT HDMS

All mass spectrometry measurements described within this study were performed using a **Waters SYNAPT HDMS**, which is a hybrid quadrupole-ion mobility-orthogonal acceleration TOF instrument (oa-TOF). This instrument has multiple

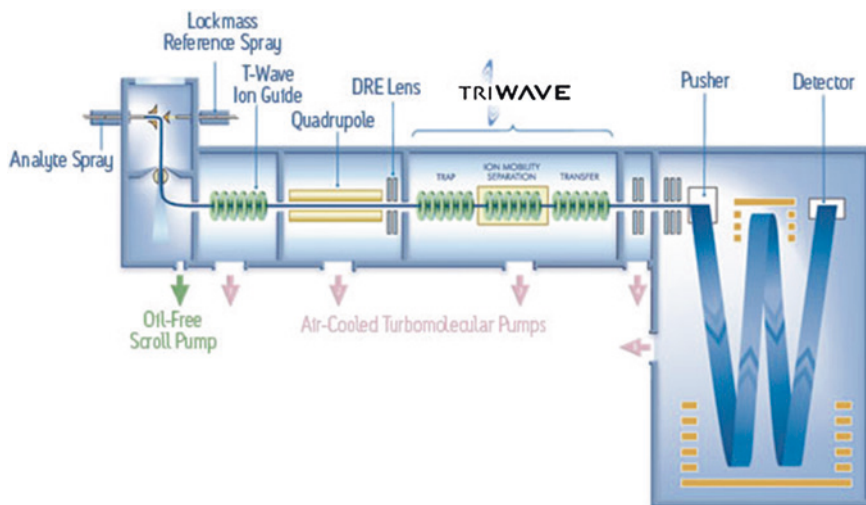


Fig. 2.1 Schematic of a Waters SYNAPT HDMS mass spectrometer. Image taken from Waters website

ionisation capabilities, of which ESI and nano-ESI were utilised during this study. The instrument schematic shown in Fig. 2.1 highlights the position of various components within the mass spectrometer. The quadrupole is located immediately after the first ion guide, and has two modes of operation. In RF mode, the quadrupole transfers all ions into the Triwave region of the instrument. However, when DC voltages are applied to the rods, the quadrupole can be used as a mass filter (as described in Sect. 1.2.3.1). The resolving power of the quadrupole can be manually altered using the MassLynx software to generate wide/narrow isolation windows.

Collision induced activation was performed in the trap region of the instrument, which is a 10 cm cell consisting of stacked ring electrodes, and utilises an RF current to focus the ions. The cell is filled with an inert buffer gas, in this case Argon, to promote the collisional activation process. The kinetic energy applied to ions in this region is the product of the charge state and the voltage applied to accelerate the ion into the trap. This can be controlled by varying the potential difference between the first ion guide and the trap (Fig. 2.1).

Nanospray Capillaries

NanoESI capillaries were prepared from **borosilicate glass capillaries (OD = 1.00 mm, ID = 0.78 mm, length = 10 cm)** (Warner), using a **Flaming/Brown P-97 micropipette puller** (Sutter Instruments). The pulled tip was then coated in gold for electrical conductivity using a vacuum evaporator built in-house (see Sect. 2.2.9).

2.1.2 Buffers and Reagents

2.1.2.1 Reagents

Ultrapure water (18.2 M Ω ·cm), was obtained from a Millipore water purification system and was used for preparation of sample solutions. **Analytical Grade Acetonitrile (MeCN)** (Fischer Scientific) and **Trifluoroacetic Acid (TFA)** (Sigma-Aldrich) were used for all ZipTip buffers (see Sect. “Denaturing MS—ZipTip Buffers”). The **coenzyme A derivatives (acetyl-, butyryl- and malonyl)** used in assays were purchased from Sigma-Aldrich. **N-acetylcysteamine (SNAC) thioesters** were provided by the Piel lab (ETH, Zurich), and stock solutions were solubilised in **dimethyl sulfoxide (DMSO)** (Fischer Scientific). **Iodoacetamide** (Sigma-Aldrich) was used for all alkylation reactions.

2.1.2.2 Transformation and Expression Media

Luria Agar (LA) (Sigma-Aldrich) was used to grow cell colonies prior to transformations. **Millers Modification Luria Broth (LB)** (Sigma-Aldrich) was used for all cell cultures of plasmid and protein synthesis. Cell culture additives such as **Kanamycin (Kan)** (Sigma-Aldrich), **Chloramphenicol (Cm)** (Sigma-Aldrich), **Tetracycline (Tet)** (Sigma-Aldrich) and **Isopropyl-1-thio- β -D-galactopyranoside (IPTG)** (Melford) were re-suspended as concentrated stock solutions before dilution into cell culture media. Components of buffers were purchased from either Sigma Aldrich or Fischer Scientific.

2.1.2.3 Expression Vectors

All expression vectors were supplied by Professor Jörn Piel (ETH, Zurich). Each vector contains the cDNA sequence for the protein domain of interest. Expression conditions are detailed in Table 2.1.

2.1.2.4 Protein Purification Buffers and Reagents

All reagents in this section were purchased from Fischer Scientific or Sigma Aldrich. The compositions of buffers used for various aspects of protein purification are listed below:

Re-suspension/Binding Buffer: 25 mM Tris HCl, 500 mM NaCl, pH 7.6

Eluting Buffer: 25 mM Tris HCl, 500 mM NaCl, 500 mM Imidazole, pH 7.6

Stripping Buffer: 25 mM Tris HCl, 500 mM NaCl, 50 mM EDTA, pH 7.6

Nickel Sulphate: 100 mM NiSO₄

SDS-PAGE Running Buffer: 25 mM Tris HCl, 192 mM Glycine, 0.1 % SDS

TAE Buffer: 40 mM Tris HCl, 20 mM Acetic acid, 1 mM EDTA

Table 2.1 Expression conditions for recombinant proteins

Construct	Plasmid	Resistance	Inducer	Expression Temp (°C)
BaeJ KS1 (N-Term His ₈ -Tag)	pHis8-BaeKS1	Kan	1mM IPTG	16
BaeJ KS2 (N-Term His ₈ -Tag)	pHis8-BaeKS2	Kan	1mM IPTG	16
BaeL KS5 (N-Term His ₈ -Tag)	pHis8-BaeKS5	Kan	1mM IPTG	21
PsyA KS1 (N-Term His ₈ -Tag)	pHis8-PsyKS1	Kan	1mM IPTG	16
PsyA KS2 (N-Term His ₈ -Tag)	pHis8-PsyKS2	Kan	1mM IPTG	16
PsyD KS3 ⁰ (N-Term His ₈ -Tag)	pHis8-PsyKS3	Kan	1mM IPTG	16
PsyA ACP3 (N-Term His ₈ -Tag)	pHis8-ACP3	Kan	0.75mM IPTG	16
PedC AT (C-Term His ₆ -Tag)	pET-24a-PedC	Kan	1mM IPTG	18
GroES-GroEL-Tig	pG-Tf2	Cm	112μM Tet	18
PedD AT (N-Term His ₈ -Tag)	pHis8-PedD	Kan	1mM IPTG	16
PsyA ACP1-KS1 (N-Term His ₆ -Tag)	pET28b-ACPKS1	Kan	1mM IPTG	16
PPT Svp (C-Term His ₆ -Tag)	pQE-70-Svp	Kan	1mM IPTG	16

Regions highlighted in grey indicate co-expression

SDS-PAGE gels were made at 12.5 and 15 % depending upon the size of protein being analysed. Table 2.2 details the amounts of each reagent required.

2.1.2.5 Sample Preparation for Mass Spectrometry Analysis

Non-denaturing MS

All non-denaturing MS measurements were conducted in aqueous NH₄OAc (Fischer Scientific) at various concentrations ranging from 5–500 mM.

Table 2.2 Volumes of reagents required for SDS-PAGE gels

	12.5 % resolving	15 % resolving	4 % stacking
mQH ₂ O	3.4 mL	2.8 mL	3.1 mL
40 % Acrylamide	2.4 mL	3.0 mL	0.5 mL
1.5 M Tris, pH 8.8	2.0 mL	2.0 mL	1.25 mL
10 % SDS	80 μ L	80 μ L	50 μ L
10 % APS	80 μ L	80 μ L	50 μ L
TEMED	8 μ L	8 μ L	5 μ L
Total vol.	8 mL	8 mL	5 mL

Denaturing MS—ZipTip Buffers

The composition of buffers used for Zip-Tip preparation and subsequent MS-analysis is detailed below:

Equilibrium Buffer: 0.1 % TFA in mQH₂O

Elution Buffer: 20:80 mQH₂O:MeCN, 0.1 % TFA.

2.1.3 Consumables

2.1.3.1 Protein Expression and Purification

Electrocompetent cells were transformed using **MicroPulser electroporation cuvettes** (Bio-Rad). Purification of His-Tag fusion proteins was conducted using a **Hi-Trap Chelating column** (GE Healthcare), and any subsequent gel filtration steps were carried out on **Superdex 75/200 columns** (GE Healthcare) depending upon protein size. Proteins were concentrated using **Vivaspin 20 ultrafiltration spin filters** (Sartorius) with a 5 kDa or 30 kDa cut-off depending upon the size of the protein.

2.1.3.2 Mass Spectrometry

The **nanoESI capillaries** were prepared from borosilicate glass tubes 1.0 mm outer and 0.78 mm inner diameter (Warner Instruments). The pulled capillaries were then coated in gold using **99 % gold wire** (VWR International) using a home-built vacuum evaporator. Samples were loaded into capillaries using **GELoader tips** (Eppendorf). Sample preparation for denaturing-MS was conducted using **C₁₈ and C₄ ZipTip** (Millipore) pipette tips.

2.2 Methods

2.2.1 Protein Expression and Purification

2.2.1.1 Transformations

Electrocompetent *Escherichia coli* XL-1 Blue and BL-21 (DE3) were used for plasmid synthesis and protein synthesis respectively. Aliquots (100 μ L) of electrocompetent cells were thawed on ice, and 20–100 ng of plasmid was added and mixed. Cells were kept on ice for 5 min before transferring to an electroporation cuvette, where an electrical pulse is applied to the cells. Immediately following electroporation, cells were transferred to 900 μ L of LB medium and incubated at 37 °C with agitation for 1 h. Typically, 100 μ L of cells were then plated onto LA plates enriched with the appropriate antibiotic (30 μ g/mL Kanamycin or Chloramphenicol). Plates were incubated overnight at 37 °C, with transformation of the cells confirmed by presence of colonies, which were picked and grown for plasmid synthesis (XL-1 Blue) or protein synthesis (BL-21 (DE3)).

2.2.1.2 Protein Overexpression

A single colony of transformed *Escherichia coli* BL-21 (DE3) was selected and added to LB media (10 mL) containing kanamycin (30 μ g/mL) and incubated overnight at 37 °C. The resulting pre-culture was added to LB media (1 L) containing kanamycin (30 μ g/mL), followed by incubation at 37 °C (see Table 2.1). Protein expression was induced by addition of IPTG (1 mM) when the optical density of the culture reached 0.6, and expression was allowed to proceed overnight at an optimized temperature (see Table 2.1). Following expression, cells were collected by centrifugation (4,000 $\times$ g, 15 min, 4 °C) and re-suspended in buffer (25 mM Tris-HCl, 500 mM NaCl, pH 7.6) at 5 mL/L of growth.

2.2.1.3 Purification of PHis8-Fusion Proteins

Escherichia coli BL-21 (DE3) cells containing overexpressed pHis8-fusion proteins were lysed by sonication. The lysate was then centrifuged (37,000 $\times$ g, 30 min, 4 °C). The resulting supernatant was loaded onto a HiTrap® Chelating Column (GE Healthcare), pre-loaded with 100 mM NiSO₄ and equilibrated in re-suspension buffer. An initial wash of 5 % eluting buffer (25 mM Tris-HCl, 500 mM NaCl, 25 mM Imidazole, pH 7.6) was applied to remove contaminating proteins. A gradient of 5–100 % of eluting buffer was applied over a volume of 60 mL to elute the pHis8-fusion domain. Presence of protein in fractions was confirmed by SDS-PAGE, and an additional gel filtration step (Superdex 75/200 GE Healthcare) was applied in cases where excessive contamination was observed.

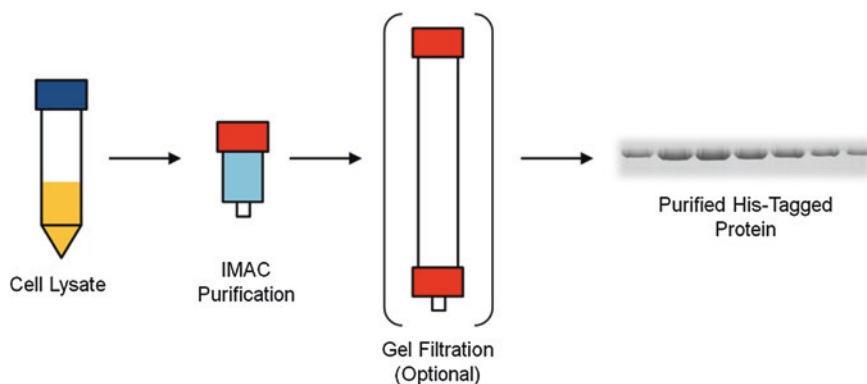


Fig. 2.2 Principle of using His-Tag proteins and IMAC chromatography columns to purify proteins. The matrix of the HiTrap[®] Chelating Column forms a tetradentate complex with Ni^{2+} . His-Tagged proteins can then provide the final two bonds to form the stable hexadentate complex, and immobilising the protein on the column

Protein-containing fractions were pooled and concentrated using a Viva-Spin centrifugal concentrator at an appropriate MWCO (5 kDa for ACP, 30 kDa for KS). Addition of 10 % glycerol (v/v) to the resulting concentrate of pure protein allowed snap-freezing in liquid N_2 , followed by storage at -80°C (Fig. 2.2).

2.2.1.4 Site-Directed Mutagenesis

BaeJ KS1 (N206A), (M268A), (L450A) and BaeL KS5 (M237A)

The mutant KS1 and KS5 genes were constructed using the Phusion (New England Biolabs) mutagenesis procedure. The entire expression plasmid, pHis8-BaeKS1, was amplified as a linear product using the primers in Table 2.3, which had been previously phosphorylated using polynucleotide kinase (New England Biolabs). The PCR product was digested with DpnI to remove the template DNA, gel purified from a TAE-Agarose gel using a Nucleospin[®] gel purification kit (Macherey-Nagel), ligated overnight using T4 Ligase (New England Biolabs) and transformed into *E. coli* strain XL1-Blue. The presence of the correct mutation was confirmed by sequencing.

BaeJ KS1 (C207A) and PedD(R97Q)

Site directed mutagenesis for the BaeJ KS1 (C207A) and PedD(R97Q) mutants were performed using a Stratagene Quikchange II kit following manufacturers protocol, using pHis8-BaeKS1 and pHis8-PedD respectively as a templates. Oligonucleotide primers used are shown below in Table 2.4.

Table 2.3 Oligonucleotide primers for Bae KS1 and KS5 mutants

Mutant	Primers	Mutation
KS1(N206A)	KS1N206A_For (5'-TGCTCATCTTCGTTAATCGGCCTGC-3') KS1N206A_Rev (5'-TGCGCGTGGACAAAATAGCTCGG-3')	AAC → GCA (Asn → Ala)
KS1(M268A)	KS1M268A_For (5'-GCCATCGGCGGTGAAGGGG-3') KS1M268A_Rev (5'-GCCGTCAGCATCCGCGTCAAACG-3')	ATG → GCC (Met → Ala)
KS1(L450A)	KS1L450A_For (5'-GCCGGCGGAACAAACACACACG-3') KS1L450A_Rev (5'-GCCGAAAGAGCTTAAAGCCATGCGG-3')	CTT → GCC (Leu → Ala)
KS5(M237A)	KS5MA_For (5'-TGCTCTTCTTTAACAGCCATTCATCTGG-3') KS5MA_Rev (5'-TGCCGTATCCAGAGCCATGCTCG-3')	ATG → GCA (Met → Ala)

Table 2.4 Oligonucleotide primers for Bae KS1(C207A) and PedD(R97Q) mutants

Mutant	Primers	Mutation
KS1(C207A)	KS1C207A_For (5'-CTATTTGTCCACGCCAAC <u>CGC</u> CTCATCTTCGTTAATCGGC-3') KS1C207A_Rev (5'-GCCGATTAACGAAGATGAG <u>GCG</u> GTTGGCGTGGACAAAATAG-3')	AAC → GCA (Cys → Ala)
PedD(R97A)	PedDR97Q_For (5'-CTGAGACTCGTGCAGAAGC <u>AG</u> GGTGATCTGATGAGTCAGG-3') PedDR97Q_Rev (5'-CCTGACTCATCAGATCACC <u>CT</u> GCTTCTGCACGAGTCTCAG-3')	CGT → CAG (Arg → Gln)

Table 2.5 Oligonucleotide primers for PsyA ACP3(Δ37,38)

Mutant	Primers	Mutation
PsyA ACP3(Δ37,38)	ACP_For(5'-GCGTAGAGGATCGAGATCTCG-3') ACP_Rev (5'-GCATAAGCTTATACCGCTTCGAGCTGC-3')	ΔCys, Val

PsyA ACP3(Δ37,38)

The PsyA ACP3(Δ37,38) construct was amplified from the plasmid pHis8-ACP3 which contained full-length ACP3 using the primers shown in Table 2.5. ACP_For bound upstream of the ACP3 start codon and incorporated a *Bgl*III restriction site present in the pHis8-ACP3 vector sequence into the PCR product. ACP_Rev replaced the Cys37 codon with an in-frame stop codon and incorporated a *Hind*III restriction site. The PCR product was digested with *Bgl*III and *Hind*III, separated on a TAE-Agarose gel, excised and purified with a Genejet gel extraction kit (Fermentas). The plasmid pHis8-ACP3 was also digested with *Bgl*III and *Hind*III,

Table 2.6 Oligonucleotide primers for PsyA ACP1-KS1

Construct	Primers
PsyA ACP1-KS1	ACPKS1_For (5'-GCGTAGAGGATCGAGATCTCG-3')
	ACPKS1_Rev (5'-GCATAAGCTTATACCGCTTCGAGCTGC-3')

separated on a TAE-Agarose gel, and the band corresponding to the vector fragment was excised and purified with a Genejet gel extraction kit (Fermentas). The purified *BglIII*–*HindIII* PCR product was ligated into the purified *BglIII*–*HindIII* pHis8-ACP3 vector fragment using T4 ligase (New England Biolabs), transformed into XL1-blue competent cells (see Sect. 2.2.1.1), and plasmids encoding PsyA ACP3(Δ 37,38) was confirmed by sequencing.

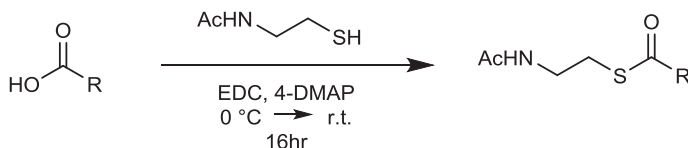
Cloning of PsyA ACP1-KS1 Didomain

The DNA sequence containing ACP and KS1 was amplified from the pPSKF1 fosmid kindly provided by Dr. Anna Vagstad (Piel lab, ETH Zurich), using the primers in Table 2.6, and conducted by José Afonso. These primers were designed in interdomain regions in locations rich in hydrophilic residues and without specific secondary structure, as predicted the PHYRE2 server [1]. The forward primer included an NdeI restriction site while the reverse primer included a Stop codon and a NotI restriction site. The PCR amplification reaction was composed of PCR reaction buffer containing Mg^{2+} , diluted from a 10x concentrated stock (Roche), 50 ng template DNA, 200 μ M of each dNTP, 0.25 μ M of each primer and 5 U of Taq DNA Polymerase (New England Biolabs), in a total volume of 50 μ l.

The amplification product was cleaned using the GeneJET PCR Purification kit (Thermo Scientific) and simultaneously digested with NdeI and NotI (New England Biolabs) for 3 h to produce sticky ends. The pET28b plasmid was also double digested with these two enzymes to cut the corresponding restriction sequences within the multiple cloning site. The digested amplification product and the open pET28b vector were then loaded onto a 1 % agarose gel and purified using the Genejet gel extraction Kit (Thermo Scientific). The insert and vector were mixed with final concentrations of 40 and 120 μ M, respectively, in T4 DNA ligase buffer (New England Biolabs), diluted from a 10x stock solution. For 20 μ l of this reaction mixture, 400 U of T4 DNA ligase (New England Biolabs) was added and the sample was incubated overnight at room temperature. The sample was then used to transform XL1 blue cells.

2.2.2 Synthesis of *N*-Acetylcysteamine Thioesters

SNAC compounds were obtained from the Piel Lab, ETH, Zurich. The general synthetic procedure for compounds was as follows. A solution of the



Scheme 2.1 General coupling methodology for the synthesis of acyl-*N*-acetylcysteamine thioesters from *N*-acetylcysteamine and corresponding acid, using DCC and 4-DMAP at room temperature for 16 h

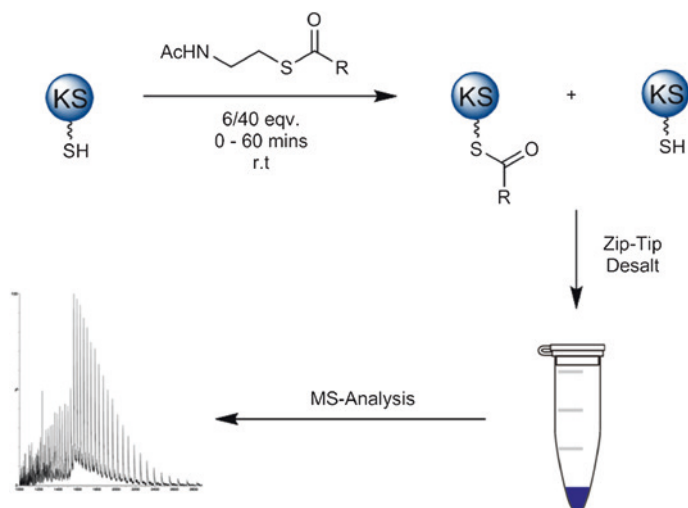
corresponding acid (5 mmol) in dichloromethane (15 mL) was cooled to 0 °C. 4-Dimethylaminopyridine (4-DMAP, 1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC, 6 mmol) and *N*-acetylcysteamine (6 mmol) were added, and stirred overnight at 25 °C. The reaction was then quenched with saturated aqueous ammonium chloride and extracted with dichloromethane, followed by drying with MgSO_4 and purification by column chromatography (Scheme 2.1). Characterisation of all compounds can be found in the following publications [2–5]. Compounds were solubilised in DMSO, from which concentrated stock solutions were made, and further diluted when added to incubation reactions (5 % v/v).

2.2.3 Ketosynthase Acylation Assay

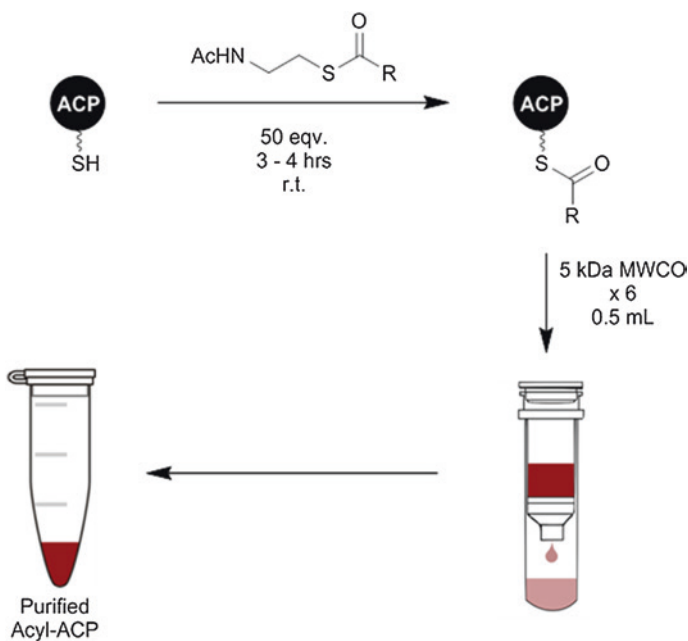
Acylation reactions were conducted in storage buffer (25 mM Tris, 500 mM NaCl, 10 % (v/v) glycerol, pH 7.6). SNAC-thioesters were incubated with KS domains at a final concentrations ranging from 0.5–2 mM for comparative substrate studies. In order to ensure the SNAC-thioesters remained in solution, the concentration of DMSO was adjusted to 5 % (v/v). Acylation reactions were allowed to run for various periods of time between 0–10 min at 25 °C, before quenching with 0.1 % TFA and ZipTip desalting (see Sect. 2.2.15.1) for MS analysis (Scheme 2.2).

2.2.4 Synthesis of Acyl–ACP Carrier Proteins

Acylation of the ACP PPant thiol was achieved by incubation of 200 μM ACP (25 mM Tris HCl, 500 mM NaCl, pH 7.4) with 10 mM SNAC thioester re-suspended in DMSO, and incubated at a 12.5 % (v/v) ratio to aid SNAC solubility. Reactions were monitored by ESI-MS at regular time intervals for the presence of the acyl-ACP adduct. Generally, acylation reactions were complete after 3–4 h at 25 °C. Removal of excess SNAC was achieved by spin filtration using 5 kDa MWCO columns (Scheme 2.3).



Scheme 2.2 Standard workflow for performing KS acylation assays followed by MS analysis



Scheme 2.3 Workflow for the synthesis of acyl-ACPs from SNAC thioesters, and subsequent purification

2.2.5 *Acyl-ACP Ketosynthase Loading Assay*

KS loading assays were conducted in storage buffer (25 mM Tris, 500 mM NaCl 10 % (v/v) glycerol, pH 7.6). Typically, 20 μ M KS was incubated with 100 μ M acyl-ACP and the reaction was allowed to proceed at 25 °C. Aliquots were removed at various time points between 2–32 h, and subsequent ZipTip clean-up of the samples allowed MS analysis of each time point (see Sect. 2.2.15.1).

2.2.6 *PedC/PedD(R97Q) Hydrolase Assays*

Hydrolase assays were conducted in storage buffer (25 mM Tris, 500 mM NaCl, 10 % v/v glycerol, pH 7.4) in the presence of GroEL. Typically, PedC (5 μ M) was incubated with acyl-ACPs (20 μ M) in a 30 μ L reaction for various periods of time ranging from 0.5–60 min at 25 °C. The reaction was quenched using 0.1 % TFA, followed by ZipTip desalting for MS analysis (see Sect. 2.2.15.1).

2.2.7 *Acyltransferase Extender Unit Specificity Assays*

Stocks of malonyl- methylmalonyl- and acetyl-CoA were made to 20 mM in milliQ H₂O, snap frozen and kept at –80 °C. Incubations with AT domains were conducted in storage buffer (25 mM Tris, 500 mM NaCl, 10 % v/v glycerol, pH 7.6) with addition of required amount of extender unit. Reactions were allowed to run for 10 min at 25 °C, before quenching with 0.1 % TFA and ZipTip desalting (see Sect. 2.2.15.1) for MS analysis.

2.2.8 *PedD Malonyl Loading and Unloading of ACP*

PedD catalysed malonyl loading and unloading of the ACP was conducted in storage buffer (25 mM Tris, 500 mM NaCl, 10 % v/v glycerol, pH 7.6). In the case of malonyl loading, 80 μ M PedD was incubated with 4 equivalents (320 μ M) of malonyl-CoA and allowed to react for 10 min at 25 °C. Excess malonyl-CoA was removed by spin filtration using 30 kDa MWCO columns. The resulting retentate was then incubated with 40 μ M *holo*-ACP at a 1:1 molar ratio. The transfer reaction was allowed to proceed for 20 min, with aliquots removed at various time intervals, quenched with 0.1 % TFA and desalted using a C₄ ZipTip (see Sect. 2.2.15.1). The unloading reaction was conducted under exactly the same conditions, except PedD was not pre-loaded with malonyl. The ACP was pre-loaded malonyl following the procedure detailed in Sect. 2.2.9.

2.2.9 Ketosynthase Elongation Assay

The ketosynthase-catalysed chain elongation assay described in Chap. 6 required multiple preparative synthetic steps before the final assay could be conducted. This section details the required synthetic steps and the final assay procedure.

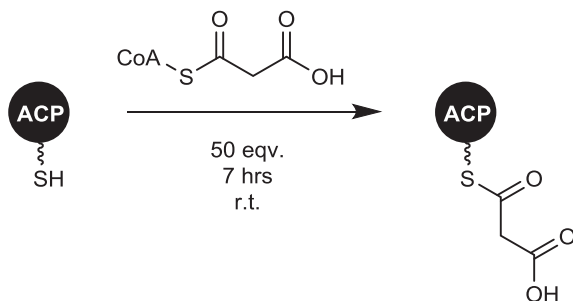
2.2.9.1 Synthesis of Malonyl-ACP

Malonylation of the ACP PPant thiol was achieved by incubation of 200 μ M ACP (25 mM Tris HCl, 500 mM NaCl, pH 7.4) with 8 mM malonyl-CoA (Scheme 2.4). Reactions were monitored by ESI-MS at regular time intervals for the presence of the malonyl-ACP adduct. Reactions were complete after 7 h at 25 $^{\circ}$ C.

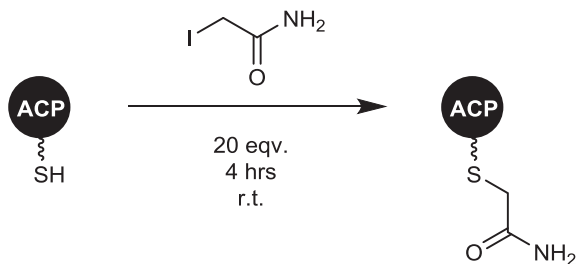
2.2.9.2 Synthesis of Alkyl-ACP

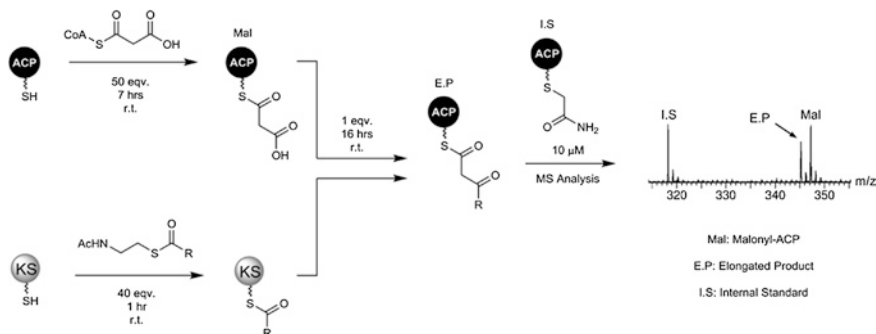
The synthesis of alkyl-ACP, for use as an internal standard, was achieved by incubation of 200 μ M ACP (25 mM Tris HCl, 500 mM NaCl, pH 7.4) with 4 mM iodoacetamide, in the absence of light (Scheme 2.5). Reactions were monitored by ESI-MS at regular time intervals for the presence of the alkyl-ACP adduct. Alkylation reactions were complete after 4 h at 25 $^{\circ}$ C, and excess iodoacetamide was removed by spin filtration using 5 kDa MWCO columns.

Scheme 2.4 Synthesis of malonyl-ACP from malonyl-CoA and *holo*-ACP



Scheme 2.5 Synthesis of alkyl-ACP from iodoacetamide and *holo*-ACP, in the absence of light





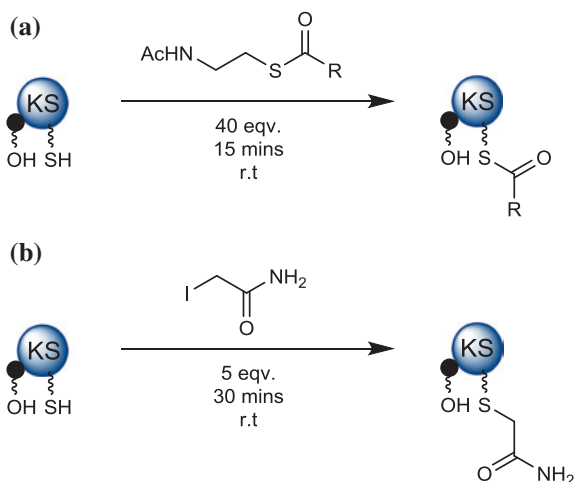
Scheme 2.6 Overview of the MS-based elongation assay for probing the substrate specificity of KS domains using a range of *N*-acetylcysteamine thioesters and malonyl-ACP. Following incubation of acyl-KS with malonyl-ACP the reaction is quenched and alkylated ACP added as an internal standard. MS/MS analysis reveals the extent of elongation by detecting and quantifying β -ketoacyl-ACP

2.2.9.3 Ketosynthase Elongation Reactions and MS Analysis

KS elongation assays were conducted in storage buffer (25 mM Tris, 500 mM NaCl, 10 % v/v glycerol, pH 7.4). Typically, the KS was pre-acylated by incubation with SNAC thioester (4 mM) for 2 h, followed by concentration to 100 μ M using a spin concentrator. Malonyl-ACP was produced by incubation of PsyA ACP3(Δ 37,38) (100 μ M) with malonyl-CoA (8 mM) for 6 h at 25 $^{\circ}$ C. The resulting acyl-KS and malonyl-ACP solutions were subsequently mixed in a 1:1 ratio yielding a reaction solution containing 50 μ M acyl-KS and 50 μ M malonyl-ACP. The reaction was allowed to proceed for 16 h, followed by addition of 10 μ M alkyl-ACP and subsequent ZipTip desalting and MS analysis (Scheme 2.6). During MS analysis, the 7⁺ charge state of the ACP was isolated (m/z = 1579) with a low quadrupole resolution to encompass all species of this charge state. Activation was achieved by applying collision energy of 20 V to the trap region of the instrument.

2.2.10 Monitoring Acyl-Transfer in PsyA ACP1-KS1

The ability to monitor the location of an acyl chain in the PsyA ACP1-KS1 domain required several pre-synthesised species. Acyl-ACP1-KS1 was used as a starting point for reverse transfer assays, and alkyl-ACP1-KS1 was used to inactivate the active site Cys of the KS, therefore acting as a control for subsequent reactions. In this section, the synthesis of these species is described in addition to the MS-based assay.



Scheme 2.7 Synthesis of **a** Acyl-PsyA ACP1-KS1 using SNAC thioesters and **b** Alkyl-PsyA ACP1-KS1 under mild conditions using iodoacetamide in the absence of light

2.2.10.1 Acylation and Alkylation of PsyA ACP1-KS1

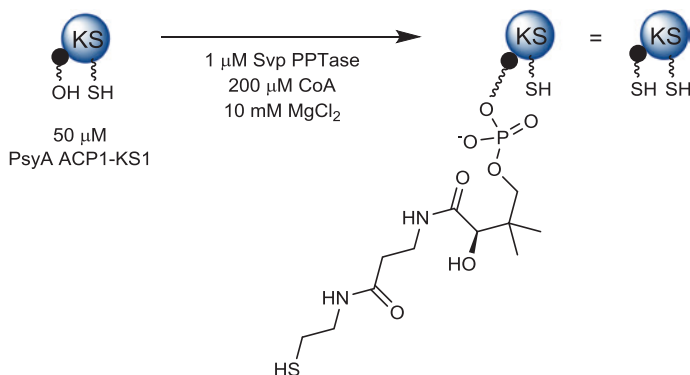
Acylation of PsyA ACP1-KS1 was conducted in a similar manner to that of the ketosynthase acylation assay described in Sect. 2.2.3, followed by removal of excess SNAC using 30 kDa MWCO spin filters (Scheme 2.7a). Alkylation of the KS active-site Cys from the ACP1-KS1 didomain was performed under mild conditions to alkylate the active site Cys residue only, by incubation with 5 equivalents of iodoacetamide in the absence of light (Scheme 2.7b).

2.2.10.2 Phosphopantetheinylation of PsyA ACP1-KS1

The PPTase-catalysed phosphopantetheinyl loading of PsyA ACP1-KS1 was carried out in accordance with previously published methods [6]. Minimal amounts of Svp PPTase from *Streptomyces verticillus* were used to reduce the complexity and heterogeneity of the subsequent mass spectra. Optimised conditions allowed 1 μ M Svp PPTase to be used for complete loading of 50 μ M PsyA ACP1-KS1, in the presence of 10 mM MgCl₂ and 200 μ M CoA (Scheme 2.8).

2.2.10.3 Monitoring Reverse Acyl-Transfer in PsyA ACP1-KS1

Pre-acylated *apo*-ACP1-KS1 was prepared as described in Sect. 2.2.12. Subsequent addition of Svp PPTase, CoA and MgCl₂ generated



Scheme 2.8 The enzymatic attachment of phosphopantetheine from co-enzyme A to a conserved serine residue on *apo*-ACP1-KS1, catalysed by the Svp PPTase

holo-acyl-ACP1-KS1, which was allowed to equilibrate for 10 min. The resulting reaction was then desalted using a C₄ ZipTip into 80:20 MeCN: mQH₂O, 0.1 % TFA for MS analysis. The location of the acyl unit was monitored using the PPant ejection assay, isolating the 73⁺ charge state of *holo*-acyl-ACP1-KS1 and application of 12 V in the trap region of the MS. Measurement of the ratio between unmodified PPant (*m/z* 261.2) and acyl-PPant species allowed the location of the acyl chain to be interrogated (Scheme 2.9).

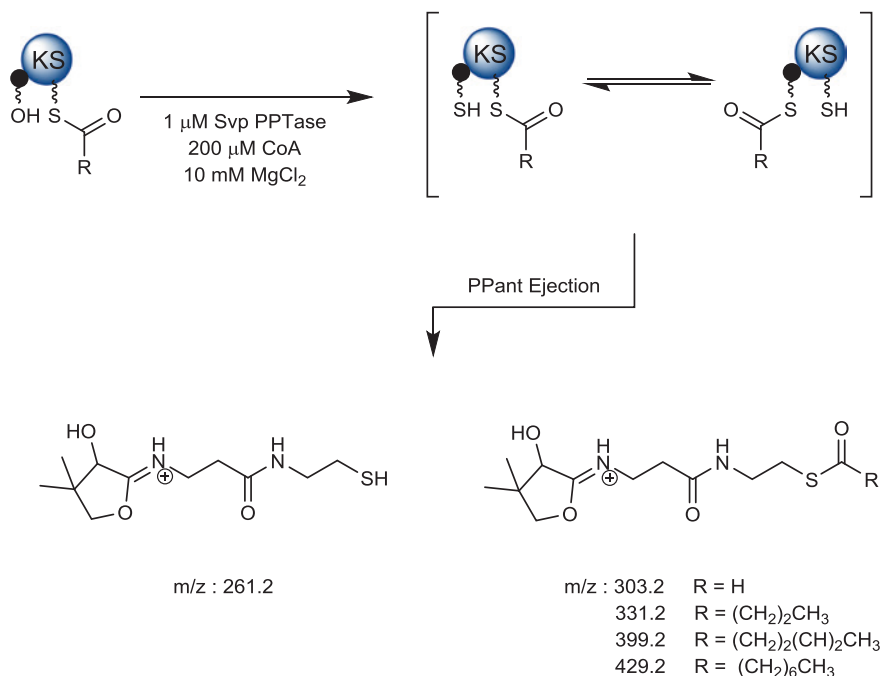
2.2.11 Sample Preparation for Mass Spectrometry

2.2.11.1 Zip-Tip Desalting

Both C₁₈ and C₄ ZipTips™ were used to prepare samples; the C₁₈ was primarily used for desalting of the ACP, whereas C₄ was used for KS and AT domains. The ZipTip was washed with two 10 μL aspirations of 50 % MeCN, followed by five 10 μL aspirations of H₂O/0.1 % TFA solution. The protein sample was then loaded onto the ZipTip column by 15–20x 10 μL aspirations of the sample, each time injecting back into the sample vial. The loaded protein sample was then desalted by x15 10 μL aspirations of 0.1 % TFA, followed by elution of the sample into 8 μL H₂O/80 % MeCN/0.1 % TFA.

2.2.11.2 Viva-Spin MWCO Desalting

Proteins sprayed from non-denaturing conditions required buffer exchanging into NH₄OAc. This was achieved by x5 concentration cycles at 11,500 xg, from 0.5 mL → 50 μL using Vivaspin 500 ultrafiltration columns (Sartorius).



Scheme 2.9 Overview of the MS-based method for monitoring acyl transfer within PsyA ACP1-KS1. Following loading of CoA-SH or acyl-CoA, the PPant ejection assay is used to identify the location of the acyl unit

2.2.12 Pulling and Gold-Coating Nanospray Capillaries

Nano-ESI capillaries were pulled using a Flaming/Brown P-97 micropipette puller (Sutter Instruments). In order to achieve a tip shape shown in Fig. 2.3, the parameters were carefully optimised by to obtain a satisfactory tip shape [7]. Although parameters can change when using different filaments, a typical set of values are detailed in Table 2.7.

Once pulled, the capillaries were coated in gold using a home-built vacuum evaporator, depicted in (Fig. 2.4). Pre-pulled capillaries were placed into a supporting rack and placed in the evaporation chamber, directly underneath the evaporation boat. Gold wire (~ 20 mg) was then placed into the evaporation boat, and a vacuum ($\sim 1.2 \times 10^{-4}$ mbar) was applied to the chamber. Once a suitable vacuum was achieved, 50 V was applied to the evaporation boat using an autotransformer and evaporation was allowed to proceed. Following evaporation (typically 2 min), the autotransformer was set to 0 V, and the chamber was vented using the vent valve. This procedure was performed twice to coat each side of the capillaries.

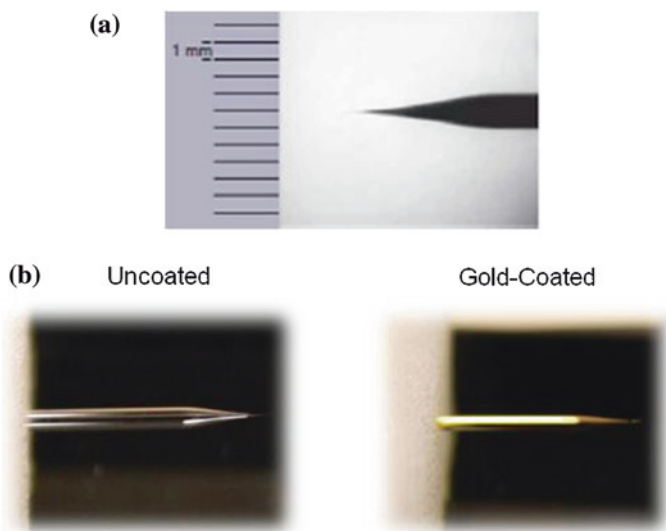


Fig. 2.3 **a** Microscope view of optimal tip shape for nESI capillaries. Taken from [4]. **b** Photographs of nESI capillaries pulled in-house, before and after gold coating

Table 2.7 Instrument parameters for Flaming/Brown P-97 micropipette puller

Heat	750	x3 cycles
Time	80	
Pull velocity	15	

2.2.13 Mass Spectrometry Instrument Parameters

Instrument parameters were optimised for each experiment to provide the highest relative signals. For experiments requiring collision induced activation, the voltage applied in the trap region was optimised such that minimal secondary fragmentation of ejected ions occurred (Table 2.8).

2.2.14 Calculation of Acyl-KS Concentrations Using MS

Denatured KS spectra obtained from acylation experiments were subjected to minimal smoothing and noise reduction. The spectra were deconvoluted using the transform function of MassLynx, taking an average of all charge states. Relevant peak intensities were recorded and converted into the concentration of [KS-SH] and [KS-acyl] respectively. Data was recorded in triplicate, and an average taken for kinetic plots.

Fig. 2.4 Photograph of home-built vacuum evaporator. Components are labelled as follows:

a Evaporation chamber.
b Evaporation boat. **c** Vent valve. **d** Autotransformer.
e Vacuum gauge controls and read-out



Table 2.8 Mass spectrometry parameters for analysis of protein domains

	KS and AT domains	ACP domain
<i>Voltages</i>		
Capillary voltage (kV)	1.5	1.5
Sample cone voltage (V)	20	10
Extraction cone voltage (V)	5	5
Trap collision voltage (V)	6	6–20 [†]
Transfer collision voltage (V)	5	5
<i>Pressures</i>		
Backing pressure (mbar)	1.6–1.8	
Trap pressure (mbar)	1.4×10^{-2}	
TOF pressure (mbar)	1.5×10^{-6}	
<i>Quadrupole profile</i>		
m/z	Dwell time (% scan time)	Ramp time (% scan time)
800	10	30
1500	40	10
3000	—	—

[†]Activation energies varied between experiments. For experiments detailed in Sect. 2.2.11, an activation energy of 20 V was applied to the 7⁺ charge state of PsyA ACP3. For experiments detailed in Sect. 2.2.11, an activation energy of 12 V was applied to the 73⁺ charge state of PsyA ACP1-KS1

2.2.15 Structure Prediction

2.2.15.1 Homology Modelling of Ketosynthase Domains

Homology models of all KS domains in this study were constructed using the CPHmodel server [8], using published KS structures 2QO3 and 2HG4 as templates [9, 10]. Further refinement of the model was achieved by energy minimisation using the YASARA server [11]. Ramachandran plots were produced using the RAMPAGE server to assess the quality of the models (<1 % of residues were found to lie outside the favoured regions in all models, and none of these were close to the active site) [12]. In the case of WT BaeL KS5, the full length biosynthetic intermediate of KS5, including the phosphopantetheine chain, was constructed in the PRODRG server [13]. A similar substrate was generated for BaeL KS5(M237A), which included an additional β -methyl branch. Both substrates were manually docked into the binding site of each domain, and re-submitted to the YASARA energy minimisation server. Homology models with acyl chains attached to the active site Cys were produced using the crystal structure of a FAS KS domain with dodecanoic acid bound as a template [14].

2.2.15.2 Homology Modelling of PedC and PedD

Homology models of PedC and PedD domains were generated using the CPHmodel server, using published AT structures 3RGI and 3BSM as templates [15, 16]. Ramachandran plots were produced using the RAMPAGE server to assess the quality of the models. Addition of a malonyl unit to the active site Ser was achieved by alignment of the respective homology models to the *E.coli* FabD, 2G2Z, structure complexed with malonyl and CoA [17].

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