1 Inhibiting *Mycobacterium tuberculosis* CoaBC by targeting a new 2 allosteric site.

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29   **Abstract**

30   Coenzyme A (CoA) is a fundamental co-factor for all life, involved in numerous
 31   metabolic pathways and cellular processes, and its biosynthetic pathway has raised
 32   substantial interest as a drug target against multiple pathogens including *Mycobacterium*
 33   *tuberculosis*. The biosynthesis of CoA is performed in five steps, with the second and
 34   third steps being catalysed in the vast majority of prokaryotes, including *M.*
 35   *tuberculosis*, by a single bifunctional protein, CoaBC. Depletion of CoaBC was found
 36   to be bactericidal in *M. tuberculosis*. Here we report the first structure of a full-length
 37   CoaBC, from the model organism *Mycobacterium smegmatis*, describe how it is
 38   organised as a dodecamer and regulated by CoA thioesters. A high-throughput
 39   biochemical screen focusing on CoaB identified two inhibitors with different chemical
 40   scaffolds. Hit expansion led to the discovery of potent inhibitors of *M. tuberculosis*
 41   CoaB, which we show to bind to a novel cryptic allosteric site within CoaB.

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54 **Introduction**

55 Tuberculosis (TB) is the most prevalent and deadly infectious disease worldwide and
 56 remains a global epidemic. Despite the availability of treatment, this disease, caused by
 57 *Mycobacterium tuberculosis*, still claims 1.5 million lives each year ¹. Current treatment
 58 regimens are long, which presents an obstacle for patient adherence and imposes a
 59 heavy social and economic burden on countries with a high incidence of TB. It is
 60 therefore critical to explore novel targets and find new and more effective drugs to
 61 combat this disease.

62 Coenzyme A (CoA) is an essential and ubiquitous cofactor involved in numerous
 63 metabolic pathways with a large number of different enzymes requiring it for their
 64 activity². CoA is essential for the synthesis of phospholipids, fatty acids, polyketides,
 65 and non-ribosomal peptides, for the operation of the tricarboxylic acid cycle and in the
 66 degradation of lipids ³. The importance of CoA for essential post-translational
 67 modifications of proteins is also well established in both eukaryotes and prokaryotes,
 68 with various proteins post-translationally modified by thioester derivatives of CoA
 69 (acylation) or CoA itself (phosphopantetheinylation and CoAlation), while several other
 70 post-translational modifications depend indirectly on CoA through the mevalonate
 71 pathway ⁴⁻⁷. Furthermore, dephospho-CoA, an intermediate of the CoA pathway, is
 72 incorporated into some RNA transcripts during transcription initiation thereby serving
 73 as a non-canonical transcription initiating nucleotide ⁸. These RNA modifications have
 74 functional consequences and occur in both eukaryotes and bacteria ⁸. In *M. tuberculosis*,
 75 CoA plays a pivotal role in the biosynthesis of complex lipids that are crucial
 76 components of the cell wall and required for pathogenicity ⁹. It is also needed for the
 77 degradation of lipids, including cholesterol, which are the primary source of energy for
 78 this organism during infection ^{10,11}. Given its ubiquitous nature, wide metabolic and

79 functional impact of its inhibition, and lack of sequence conservation between
80 prokaryotes and humans, the CoA pathway is therefore an attractive pathway for drug
81 discovery for many different infectious diseases, including TB.

82 The biosynthesis of CoA from pantothenic acid (vitamin B₅) is performed in five steps,
83 sequentially catalysed by the enzymes pantothenate kinase (CoaA, also known as
84 PanK), phosphopantothenoylcysteine synthetase (CoaB), phosphopantothenoylcysteine
85 decarboxylase (CoaC), phosphopantetheine adenylyltransferase (CoaD) and dephospho-
86 CoA kinase (CoaE). However, in the vast majority of prokaryotes, including *M.*
87 *tuberculosis*, CoaB and CoaC are encoded by a single gene to produce a fused
88 bifunctional enzyme (CoaBC). Transcriptional silencing of individual genes of the CoA
89 biosynthetic pathway of this pathogen identified CoaBC as uniquely bactericidal within
90 the CoA pathway, highlighting it as a good candidate for drug discovery¹².

91 CoaBC converts 4'-phosphopantothenate to 4'-phosphopantetheine in three steps. First,
92 4'-phosphopantothenate (PPA) reacts with CTP to form 4'-phosphopantothenoyl-CMP
93 with the release of pyrophosphate. This intermediate subsequently reacts with cysteine
94 to form 4'-phosphopantothenoylcysteine (PPC) with the release of CMP, with these two
95 steps being catalysed by CoaB. The product of CoaB is then decarboxylated by CoaC,
96 an enzyme of the homo-oligomeric flavin-containing decarboxylase (HFCD) protein
97 family, to 4'-phosphopantetheine. X-ray crystal structures have been reported for the
98 individual CoaB and CoaC enzymes in several organisms, including a structure of CoaB
99 from *Mycobacterium smegmatis*, a close relative of *M. tuberculosis*. However, a
100 structure of a full-length bifunctional CoaBC had not been determined.

101 Here we report the structure of the bifunctional CoaBC of *M. smegmatis* at 2.5 Å. We
102 identify a previously unknown allosteric site in CoaB and crucially, we report the
103 discovery of the first *M. tuberculosis* CoaBC allosteric inhibitors. Using X-ray

crystallography and enzyme kinetic experiments, we define the mode of binding of one of the inhibitors and show its impact on the protein structure and function. These results further illustrate the potential of CoaBC as a novel drug target in *M. tuberculosis*.

Results

Overall structure of CoaBC

As the HFCD protein family of flavin-binding proteins are known to form homo-oligomers¹³, we performed native electrospray-ionization mass spectrometry (ESI-MS) to investigate the stoichiometry of CoaBC, previously proposed to form a dodecamer¹³. Both *M. tuberculosis* CoaBC (MtbCoaBC) (Figure S1A) and *M. smegmatis* CoaBC (MsmCoaBC) (Figure S1B) exclusively exhibited a dodecameric assembly, with no other oligomeric species observed in the spectra, which is consistent with a strong interaction between the subunits of the complex. The dodecamer of MtbCoaBC was centred around the 56+ charge state, with an observed mass of 537 kDa, while the dodecamer of MsmCoaBC was centred around the 52+ charge state, with an observed mass of 523 kDa. These masses are 1-2% higher than the expected masses of 525 and 518 kDa for MtbCoaBC and MsmCoaBC respectively, which can be attributed to the non-specific binding of solvent molecules or ions to the protein complexes under the soft ionization conditions employed.

Structures of a few proteins of the HFCD family have been determined¹⁴⁻¹⁸. All of these structures show either a homo-trimeric or homo-dodecameric arrangement of the flavin-containing Rossmann-fold with trimers forming at each of the vertices of the tetrahedron in the case of a dodecameric arrangement¹⁵. However, all of these proteins, unlike CoaBC, contain only a single functional domain. We solved the structure of MsmCoaBC (PDB: 6TGV) at 2.5 Å resolution (Figure 1A), in the presence of CTP and

129 FMN (Figure 1B, S2A and S2B), using crystals belonging to the $H3_2$ space group with
 130 an asymmetric unit containing four protomers forming two CoaBC dimers. Data
 131 collection and refinement statistics are summarised in (Table S1). The final model
 132 (residues 2-412) covers both CoaC and CoaB, but densities for several residues in three
 133 loop regions in CoaB are not observed (residues 290-298, 336-342, 363-376).
 134 Nevertheless, all these residues except for 375 and 376, can be seen in the MsmCoaB
 135 X-ray crystal structure (PDB: 6TH2) that we also solved in this work at 1.8 Å. The N-
 136 terminal CoaC of MsmCoaBC (residues 1-179) forms the same type of dodecameric
 137 arrangement seen in other HFCD family proteins, such as the peptidyl-cysteine
 138 decarboxylase EpiD¹⁵, and it sits at the core of the dodecamer (Figure 1A and 1C) with
 139 the two domains connected through a small loop region (residues 180-189) that tightly
 140 interacts with both. The active site of CoaC sits at the interface between two protomers
 141 of one CoaC trimer and a protomer of an adjacent CoaC trimer with the FMN site
 142 facing inwards towards the hollow centre of the dodecamer (Figure 2A). A previously
 143 described flexible flap that encloses the reaction intermediate bound to *Arabidopsis*
 144 *thaliana* CoaC¹⁹ is also observed in some of the protomers, but in an open
 145 conformation (Figure 1B).
 146 The C-terminal CoaB of MsmCoaBC also displays a Rossmann fold consistent with
 147 several other CoaB structures solved previously, including both the eukaryotic form, in
 148 which CoaB exists as an individual polypeptide, and the bacterial form where CoaB is
 149 typically fused with CoaC²⁰⁻²². Each CoaB of MsmCoaBC (residues 190-414)
 150 dimerises with a CoaB belonging to an adjacent trimer (Figure 1C). The full protein
 151 resembles a tetrahedron with CoaB dimers positioned at the six edges and CoaC trimers
 152 at the four vertices (Figure 1A).

153 The shortest distance between a pair of CoaB and CoaC active sites is ~30 Å (Figure
154 2B). Nevertheless, a flexible loop (residues 362-377) that covers the 4'-
155 phosphopantothenate site, when this substrate binds to the enzyme²⁰, can be seen in our
156 MsmCoaB structure, extending away from the active site. A superposition of our
157 MsmCoaB dimer structure with MsmCoaBC shows the loop extending towards the
158 CoaC active site (Figure 2B). This long loop (15-16 amino acids) is present in all
159 CoaBCs (Figure S3) and it is possible that it helps channelling the substrate from the
160 CoaB to the CoaC active site.

161 The small differences (RMSD = 1.147) in overall structure of CoaB dimers in the full
162 length MsmCoaBC and the MsmCoaB crystal structure solved at 1.8 Å (Figure S4) can
163 be attributed to artefacts of crystal packing. Similarly, the CoaC structure does not seem
164 to differ between full length MsmCoaBC and the available individual CoaC structures.
165 However, when MsmCoaB (residues 186-414) is expressed alone, the protein does not
166 dimerise in solution and is inactive (not shown). This contrasts with *E. coli* CoaB,
167 which still dimerises and is functional when expressed on its own without the N-
168 terminal CoaC^{23,24}. The CoaB dimer interface is mostly conserved, but there are clear
169 differences in the dimerisation region between MsmCoaB and *E. coli* CoaB that could
170 help to explain the different observed oligomerisation patterns (Figure S3). The absence
171 of dimerisation for the MsmCoaB when expressed alone suggests that the interactions
172 between CoaC and CoaB in *M. smegmatis*, and likely all other *Mycobacteriaceae*, are
173 fundamental for CoaB dimerisation and activity. This idea is reinforced by the fact that
174 the residues located at the interface of the two enzymes (CoaB and CoaC) are well
175 conserved in all *Mycobacteriaceae* and somewhat conserved in the sub-order
176 *Corynebacterineae*, but not outside of this group (Figure S3).

177 The CoaB dimerisation region forms a β -sandwich composed of eight anti-parallel β -
 178 strands, related by 2-fold symmetry, that contacts with the active site (Figure 2C and
 179 2D). Comparison of the MsmCoaB with human CoaB reveals that the human and many
 180 other eukaryotic CoaBs ²¹ possess two extra α -helices and β -strands involved in the
 181 dimerisation interface that help stabilise the dimer in the absence of CoaC (Figure S5).
 182 The CoaB active site is enclosed by a loop that extends from the opposing protomer and
 183 is observed for the first time in this work. This loop contains a motif “K-X-K-K”, which
 184 is widely conserved in bacteria (Figure S3), with few exceptions, and each lysine either
 185 interacts directly with the triphosphate group of CTP or through highly coordinated
 186 waters (Figure 2C). Also observed for the first time is the coordination of a cation by
 187 the triphosphate group and D281 (Figure 2C and S6). While magnesium or manganese
 188 are the favoured cations for CoaB activity ²⁵, calcium is observed in our structures
 189 instead, due to the high concentration present in the crystallization condition.

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191 **CoaBC is regulated by CoA thioesters**

192 It is known that CoA biosynthesis is regulated, in many organisms, at the first step of
 193 the pathway, which is catalysed by the enzyme CoaA ³. *M. tuberculosis* and many other
 194 mycobacteria possess a CoaA (type I PanK) as well as CoaX (type III PanK). However,
 195 only the type I PanK seems to be active based on studies in *M. tuberculosis* ²⁶. CoA and
 196 its thioesters competitively inhibit *E. coli* CoaA by binding to the ATP site, with CoA
 197 being the strongest regulator ^{27,28}. Nevertheless, at physiologically relevant levels of
 198 CoA there is only a low level inhibition of CoaA ²⁸. It is also known that *M.*
 199 *tuberculosis* CoaD, the enzyme that catalyses the fourth step of the pathway, is
 200 competitively inhibited by CoA and its product dephospho-CoA ^{29,30}. However, nothing
 201 was known about the regulation of CoaBC in any organism. We therefore examined the

effect of CoA and several of its thioesters (acetyl-CoA, malonyl-CoA and succinyl-CoA) on MtbCoaBC activity, using a coupled enzymatic assay that quantifies the release of pyrophosphate (EnzChek pyrophosphate assay). Controls were performed to assess the activity of these compounds against the two coupling enzymes and the compounds showed an absence of inhibition at the tested range of concentrations. Inhibition of CoaB activity by CoA and acyl-CoAs was observed, with IC_{50} values ranging from 38 to 148 μ M, far below the predicted intracellular concentrations of acyl-CoAs³¹, with succinyl-CoA displaying the highest inhibition (Figure 3A and Table 1). Competition assays with the three substrates and acetyl-CoA show a competitive mode of inhibition relative to CTP and PPA with a K_i of 22.5 and 22.4 μ M respectively, and non-competitive inhibition for L-cysteine with a K_i of 62.5 μ M (Figure 3B and Table 2). In the absence of a crystal structure to confirm the mode of binding, these results suggest that acyl-CoAs most likely bind to the active site itself, competing directly with CTP and PPA. Interestingly, both acyl-CoAs, involved in fatty acid synthesis, as well as those involved in the TCA cycle, show inhibition of CoaB, with larger fatty acyl chains showing higher inhibition of CoaB (Figure 3A).

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219 **Identification of CoaB inhibitors using high-throughput screening**

Although the CoA biosynthetic pathway is considered an attractive target for drug discovery, CoA pathway inhibitors displaying potent whole cell activity are rare and the few CoaBC inhibitors that have been reported to date are in majority substrate mimicking^{24,32}.

In order to identify novel MtbCoaBC inhibitors, we have conducted a high-throughput screen of 215,000 small molecules targeting CoaB activity. To do this, an end-point pyrophosphate quantification assay was used (Biomol Green). The most potent hits

identified were compounds **1a** and **2a** with IC₅₀ values of 9 and 3.1 µM respectively (Table S2), originating from two different but related chemical scaffolds. A search was then performed for commercially available analogues. Testing of analogues of the initial hits resulted in the identification of more potent compounds with sub-micromolar IC₅₀ values (Table 1, Figure 4A, Table S2 and Figure S7). Of these, compounds **1b** and **2b** (Figure 4A and Table 1), with IC₅₀ values of 0.28 and 0.08 µM respectively, were identified as the most potent of the two chemical series and therefore were selected for further work.

Elucidation of the mode of inhibition

Following the identification of potent MtbCoaB inhibitors we went on to determine their mode of inhibition using kinetic assays. For this, the EnzChek coupled enzyme assay that measures the release of pyrophosphate was used. Control experiments were first performed to assess compound activity against the two coupling enzymes and the compounds were found to be inactive at 100 µM. The IC₅₀ values for the compounds against MtbCoaB were re-determined with this assay and the values obtained were in line with the primary screening assay (Table S2).

Competition experiments were then performed between the three CoaB substrates and the two most potent compounds of each chemical series (**1b** and **2b**). Compound **1b** showed uncompetitive inhibition for all substrates with a αK_i of 0.222, 0.078 and 0.173 µM respectively for CTP, PPA and L-cysteine (Figure 4B and Table 2), consistent with the compound binding preferentially when the three substrates are bound. Compound **2b** shows mixed inhibition relative to CTP with a K_i of 0.093 µM and uncompetitive inhibition for PPA and L-cysteine and PPA with a αK_i respectively of 0.062 and 0.049 µM (Figure 4C and Table 2). It is known that CoaB forms the phosphopantothienoyl-

252 CMP intermediate in the absence of L-cysteine³³ and, due to spatial constraints, it is
 253 likely that cysteine can only bind at the active site after the release of pyrophosphate.
 254 The data is therefore consistent with compound **1b** preferentially binding after L-
 255 cysteine enters the active site, for the last step of catalysis and the formation of 4'-
 256 phosphopantothienoylcysteine and CMP. However, compound **2b** shows a mixed
 257 inhibition for CTP, reflecting a slightly different mechanism of action. These results
 258 obtained for both compounds suggest the existence of an allosteric site in the CoaB
 259 moiety of MtbCoaBC.

260

261 **Structural basis for inhibition of CoaB by allosteric inhibitors**

262 In order to elucidate the binding mode of compound **1b** we used a truncation of the
 263 MsmCoaB (residues 187-414) that was previously crystallized before by others in the
 264 presence of CTP (PDB code: 4QJI) at 2.65 Å resolution. The screening for new
 265 crystallization conditions allowed us to find a new CTP containing condition that gave
 266 excellent resolution (1.8 Å). Comparison of this structure with the full length
 267 MsmCoaBC (Figure S4) showed only minor differences that can be attributed to crystal
 268 packing. Hence this crystallization system could be used to validate CoaB inhibitors
 269 binding outside of the CTP site.

270 MsmCoaB was co-crystallized with CTP in the absence of compound **1b** and overnight
 271 soaking of the crystals with this compound was performed. A co-crystal structure of
 272 MsmCoaB with compound **1b** was obtained and showed that the compound was bound
 273 to a site at the dimer interface of CoaB, in a deep cavity that is occluded when the
 274 compound is absent (Figure 5A-B and S8). Each CoaB dimer contains two of these
 275 sites, which are formed by a sandwich of eight β-strands and a long loop that contains
 276 the conserved “K-X-K-K” motif. This site opens to the active site and the inhibitor also

contacts with D281 that is involved in the coordination of the cation (Figure 5C). The opening/closing of this cryptic allosteric site is mediated by the side chain of R207 of the opposing protomer (Figure 5D) that moves 5.5 Å at the furthest point and, to a smaller extent, by the side chain of F282 that moves 2 Å. R207 has previously been shown to be critical for the second half of the reaction catalysed by CoaB, the conversion of the 4'-phosphopantothienoyl-CMP intermediate to *PPC*, with almost no conversion of the intermediate to *PPC* detected when this arginine is mutated to glutamine³³. Given the position of this arginine, it is likely that it is involved in the binding of cysteine. Despite the absence of a crystal structure with cysteine, kinetic data showing uncompetitive inhibition with cysteine is consistent with this.

This allosteric site is comprised of a large group of hydrophobic residues (I209, F282 L304 of protomer A and L203, I292, P299 and I302 of protomer B) many of which form hydrophobic interactions with compound **1b** (Figure 5C). Several π -interactions between the compound and the protein are also observed and involve D281 and F282 of protomer A and R207 of protomer B (Figure 5D). Hydrogen-bond interactions are formed with D281 and F282 of protomer A and R207 of protomer B. Water-mediated interactions are also observed for a group of residues that sit at the outer edge of the site (L203, H286 and D303) that is formed exclusively by protomer B (Figure 5C).

We propose that upon binding of L-cysteine, the R207 side chain moves towards the active site, and is likely involved in stabilizing/orienting L-cysteine to attack the phosphopantothienoyl-CMP intermediate. This movement opens the allosteric site, which allows binding of allosteric inhibitors to the newly created cavity. The allosteric inhibitors will then stabilize the enzyme in its substrate bound state with the position of R207 becoming locked by several hydrogen-bonds with the side chain of D281 of protomer A, the backbone carbonyl group of I292 and the side chain of D204 of

protomer B, but also by the π -interactions with the compound (Figure 5C). The residues around this site and crucially R207 are conserved across many microorganisms, suggesting that this allosteric site is present in most, if not all bacterial CoaBCs (Figure S3 and S9A). Interestingly, even though overall sequence identity is very low between the human CoaB and MsmCoaB (22%), the human enzyme also contains an arginine equivalent to R207 and a roughly similar interface with several conserved residues, but there are stark differences in the relative position of the residues at this site between the two enzymes (Figure S9B).

While we were not able to obtain co-crystal structures with other inhibitors, *in silico* docking helped to provide a possible explanation for the structure-activity relationship observed for series one and two. The highest-scoring docking pose of compound **1b**, the most potent inhibitor of series one, was almost identical to that observed in the co-crystal structure (Figure S10A), and the analogues for which docking was performed adopted a similar binding pose. The lower activity of compound **1a** relative to compound **1b** could be explained by the loss of water-mediated hydrogen bonds (Figure 5C, S10B), while the lower activity of compound **1c** could be explained by the loss of the carbonyl group which faces a highly electropositive area of the protein (Figure S10C). Compound **2b** is predicted to form direct hydrogen bonds at the bottom of the allosteric site, similar to those formed by compound **1b**, but also to interact directly with L203 and H286, forming extra hydrogen bonds at the top of the allosteric site (Figure 6). For compound **1b** the interactions at the top of the site are water mediated (Figure 5C). This could explain the higher potency of compound **2b**. Compounds **2c** and **2d** are also predicted to form direct hydrogen bond interactions at the top of the allosteric site, but the interactions at the bottom of the site are not as favourable due to the presence of extra hydroxyl groups (Figure S10D-F). The remaining compounds in series two, which

327 have fewer hydroxyl groups and/or hydroxyl groups in different positions, lose the
328 ability to form hydrogen bonds, consistent with the weaker inhibitory effect observed
329 (Table S2).

330

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332 **Screening of CoaBC inhibitors against *M. tuberculosis***

333 The in vitro whole cell activity of the compounds was further evaluated by their ability
334 to inhibit *M. tuberculosis* growth on different carbon sources. None of the compounds
335 exhibited activity in media containing glycerol or cholesterol as the main carbon source
336 (Table 3). We then tested whether the lack of inhibitory activity could be attributed to
337 the presence of BSA by determining the whole cell activity of the three most potent
338 inhibitors against *M. tuberculosis* in GAST/Fe minimal media. All the tested
339 compounds exhibited moderate to low activity in this media with compound **2b**
340 displaying the best activity of the three (Table 3). The observed differences in potency
341 between the enzymatic assay and whole cell activity are likely related to low compound
342 permeation, high efflux or metabolism.

343

344 **Discussion**

345 CoA is an essential co-factor ubiquitous across all domains of life. For many years, this
346 pathway has been considered an attractive drug target to develop new antibiotics against
347 a wide range of pathogens including *M. tuberculosis*³⁴. Furthermore, the recent
348 identification of CoaBC as a key fragility point in the CoA pathway of this organism¹²,
349 combined with the extremely low sequence identity with the human CoaB (25%),
350 makes this enzyme a highly attractive drug discovery target.

351 While a structure of an individual mycobacterial CoaB was available, we were aware
352 that the many questions remaining at the start of this work about the organization and
353 regulation of this bi-functional enzyme could have significant implications for drug
354 discovery. We therefore set out to obtain a full-length structure of a mycobacterial
355 CoaBC and we successfully solved the MsmCoaBC structure, which shares very high
356 sequence identity with the *M. tuberculosis* orthologue (86% full-length, 84% CoaB
357 enzyme) and hence is a valuable tool for studying *M. tuberculosis* CoaBC. The
358 organization of CoaBC is similar to other HFCD family proteins¹⁵ but unique in the
359 sense that it contains more than one domain and highlights how the arrangement of the
360 fused enzymes is essential for mycobacterial CoaB dimerisation and function. This
361 fused arrangement might also help to channel the CoaB product to the CoaC active site
362 more effectively. The human CoaB and other eukaryotic orthologues form stable dimers
363 due to the extra dimerisation region (Figure S5), but is also known in yeast that the
364 entire CoA pathway assembles into a metabolon centred on CoaC^{35,36} (known as
365 CAB3). This hints that close proximity between the different active sites of the CoA
366 enzymes is desirable and that substrate channelling of products and substrates between
367 different enzymes might be important in this pathway. It is not clear at this point if such
368 an arrangement for the entire CoA pathway is also present in bacteria.

369 Regulation of the CoA biosynthesis pathway was known to occur for other enzymes of
370 the pathway through feedback inhibition by CoA, but no information was available for
371 CoaBC. We demonstrate that both CoA, as well as several CoA thioesters regulate
372 CoaBC by inhibiting CoaB activity, and that these molecules act competitively for CTP
373 and PPA and non-competitively for L-cysteine. This is consistent with these molecules
374 binding to the CoaB active site but not to the L-cysteine sub-site. CoA and acyl-CoAs
375 inhibit both CoaA and CoaD enzymes to varying extents^{28,30,37}. However, the inhibitory

effect of CoA and its thioesters in the activity of these enzymes is lower when compared to what we observed in CoaBC and consequently the impact of the intracellular level of these molecules will be predominant in CoaBC. We therefore report a new and important mechanism of regulation of “*de novo*” CoA biosynthesis, mediated by the action of CoA thioesters on CoaBC. Since the reported intracellular levels of these molecules³¹ are normally above the observed IC₅₀, the activity of CoaBC is highly inhibited. This correlates well with previous work showing that “*de novo*” CoA biosynthesis closely matches dilution due to cell division³⁸. However, the data for intracellular concentrations of CoA and CoA thioesters, as well as CoA half-life was not obtained for mycobacteria, and both interspecies differences along with variations in growth conditions may affect these conclusions.

Although the CoA pathway and CoaBC have been the subject of many drug discovery efforts, few non-substrate-mimicking inhibitors of CoaBC have been reported²⁴. Our work identifies two related chemical scaffolds that potently inhibit the activity of the CoaB moiety of MtbCoaBC through a new cryptic allosteric site that sits in the dimer interface region of the CoaB enzyme. This site is closed in the CTP-bound structure, by the side chain of R207 a residue known to be involved in the second and final step of the reaction catalysed by CoaB – the conversion of the 4'-phosphopantothenoyl-CMP intermediate to PPC³³. Considering the role of this residue in the final step of product formation and that compound **1b** shows uncompetitive inhibition relative to all CoaB substrates, we propose that the opening of this site occurs upon binding of the final substrate L-cysteine. Currently it is not clear whether this new allosteric site is exploited by a natural ligand, as we were unable to identify such a biomolecule. Nevertheless, the conservation of residues at this site, across a variety of bacteria, indicates that this feature might be common to many, if not all, bacterial CoaBs.

401 Drug discovery against *M. tuberculosis* is rich in examples of compounds with potent
 402 activity against an essential enzyme but with a complete lack of whole cell activity due
 403 to the impermeable cell wall of this organism, efflux pumps, target modification
 404 enzymes and extensive capacity to metabolise compounds ³⁹. The modest vitro whole
 405 cell activity displayed by the CoaB inhibitors reported in this work, may relate to any of
 406 these issues. Nevertheless, the biochemical and structural data described herein further
 407 validate CoaBC as a promising novel anti-tubercular drug target by showing a new
 408 allosteric site that can be targeted by potent inhibitors.

409

410 **Materials and methods**

411 **Cloning and protein purification**

412 *M. tuberculosis* and *M. smegmatis* *coaBC* genes were amplified from genomic DNA of
 413 *M. tuberculosis* H37Rv strain, obtained from ATCC (ATCC25618D-2) and genomic
 414 DNA of *M. smegmatis* mc² 155, and cloned into a pET28a vector (Novagen), modified
 415 to include an N-terminal 6xHis-SUMO tag. The *M. smegmatis* *coaB* construct was
 416 obtained from the Seattle Structure Genomics Center for Infectious Disease. The same
 417 protein purification protocol was used for both *M. tuberculosis* and *M. smegmatis*
 418 CoaBC constructs.

419 *E. coli* BL21(DE3) containing pET28aSUMO-CoaBC was grown in 2XYT media at 37
 420 °C until an O.D.₆₀₀ = 0.6. IPTG was then added to a final concentration of 0.5 mM and
 421 the temperature changed to 18 °C for 18-20h. Cells were then harvested by
 422 centrifugation, re-suspended in 50 mM TRIS pH 8.0, 250 mM NaCl, 20% (w/v)
 423 glycerol, 20 mM imidazole, 5 mM MgCl₂ with protease inhibitors tablets (Roche) and
 424 DNaseI (Sigma). Cells were lysed with an Emulsiflex (Avestin) and the resultant cell
 425 lysate was centrifuged at 27000 g for 30 min to remove cell debris. Recombinant

426 CoaBCs were purified with a HiTrap IMAC Sepharose FF column (GE-Healthcare),
 427 equilibrated with 50 mM TRIS pH 8.0, 250 mM NaCl, 20% (w/v) glycerol and 20mM
 428 Imidazole. Elution was performed in the same buffer with 500mM Imidazole. Protein
 429 was dialysed in 25 mM TRIS pH 8 and 150 mM NaCl and the SUMO tag was cleaved
 430 overnight at 4 °C by adding Ulp1 Protease at a 1:100 ratio. CoaBC was concentrated
 431 and loaded on a Superdex 200 column equilibrated with 25 mM TRIS pH 8.0, 150 mM
 432 NaCl. Fraction purity was determined by SDS-page and the purest fractions were
 433 pooled, concentrated to ~10 mg.mL⁻¹ for MtbCoaBC and 30 mg.mL⁻¹ for MsmCoaBC,
 434 flash frozen in liquid nitrogen and stored at -80 °C.

435 *E. coli* BL21(DE3) containing the *M. smegmatis* CoaB construct with a N-terminal non-
 436 -cleavable 6xHis tag was grown and harvested as above and re-suspended in 20 mM
 437 HEPES pH 7.0, 500 mM NaCl, 20 mM imidazole, 5 mM MgCl₂ with protease
 438 inhibitors tablets (Roche) and DNaseI (Sigma). Cells were lysed with an Emulsiflex
 439 (Avestin) and cell lysate was centrifuged at 27000 g for 30 mins to remove cell debris.
 440 Recombinant *M. smegmatis* CoaB was purified with a HiTrap IMAC Sepharose FF
 441 column (GE-Healthcare), equilibrated with 20 mM HEPES pH 7.0, 500 mM NaCl and
 442 20 mM imidazole. Elution was carried out in the same buffer with 500 mM imidazole.
 443 Protein was concentrated and loaded on a Superdex 200 column equilibrated with
 444 20mM HEPES pH 7.0 and 500 mM NaCl. Fraction purity was assessed by SDS-page
 445 and the purest fractions were pooled concentrated to 22 mg. mL⁻¹, flash frozen in liquid
 446 nitrogen and stored at -80 °C.

447

448 **Native mass spectrometry**

449 Spectra were recorded on a Synapt HDMS mass spectrometer (Waters) modified for
 450 studying high masses. MtCoaBC and MsCoaBC were exchanged into NH₄OAc (500

451 mM, pH 7.0) solution using Micro Bio-Spin 6 chromatography columns (Bio-Rad). A
 452 sample volume of 2.5 μ L was injected into a borosilicate emitter (Thermo Scientific) for
 453 sampling. Instrument conditions were optimized to enhance ion desolvation while
 454 minimizing dissociation of macromolecular complexes. Typical conditions were
 455 capillary voltage 1.8–2.0 kV, sample cone voltage 100 V, extractor cone voltage 1 V,
 456 trap collision voltage 60 V, transfer collision voltage 60 V, source temperature 20 °C,
 457 backing pressure 5 mbar, trap pressure $3\text{--}4 \times 10^{-2}$ mbar, IMS (N₂) pressure $5\text{--}6 \times 10^{-1}$
 458 mbar and TOF pressure $7\text{--}8 \times 10^{-7}$ mbar. Spectra were calibrated externally using
 459 cesium iodide. Data acquisition and processing were performed using MassLynx 4.1
 460 (Waters).

461

462 **Crystallization**

463 For both full length *M. smegmatis* CoaBC and CoaB alone, the crystallization screens
 464 and optimization were performed at 18 °C using the sitting-drop vapour diffusion
 465 method. For CoaBC 300 nL of pure protein at 30 mg.mL⁻¹, pre-incubated with 3 mM
 466 CTP and 10 mM MgCl₂, was mixed in 1:1 and 1:2 (protein to reservoir) ratio with well
 467 solution using a mosquito robot (TTP labtech). Initial conditions were obtained in the
 468 Classics lite crystallization screen (Qiagen), solution 1. Crystals obtained in this
 469 condition diffracted poorly, therefore several rounds of optimization were performed.
 470 The final optimised condition consisted of 0.1 M BisTris pH 6.5, 10 mM CoCl₂ 0.8 M
 471 1,6-hexanediol. Crystals appeared after three days in both conditions. A cryogenic
 472 solution was prepared by adding ethylene glycol up to 30% (v/v) to the mother liquor.
 473 Crystals were briefly transferred to this solution, flash frozen in liquid nitrogen and
 474 stored for data collection.

475 For MsmCoaB, 200 nL of pure protein at 22-24 mg.mL⁻¹ with 10 mM CTP was mixed
 476 in 1:1 ratio with well solution using a Phoenix robot (Art Robbins). Crystals were
 477 obtained in Wizards classics III&IV (Rigaku) solution G4 consisting of 20% (w/v) PEG
 478 8000, 0.1 M MES pH 6.0 and 0.2 M calcium acetate. Crystals appeared after 2 days.
 479 To obtain ligand-bound structures, soaking was performed condition using the hanging-
 480 drop vapour-diffusion method as follows: 2 µL of a solution containing 20% (w/v) PEG
 481 8000, 0.1M MES pH 6.0, 0.2 M calcium acetate, 0.25 M NaCl 10% (v/v) DMSO and
 482 1-5 mM inhibitors was left to equilibrate against 500 µL of reservoir solution for 3 days.
 483 Crystals were then transferred to the pre-equilibrated drops and incubated for 24 h. A
 484 cryogenic solution was prepared by adding 2-methyl-2,4-pentanediol up to 25% (v/v) to
 485 mother liquor. Crystals were briefly transferred to this solution, flash frozen in liquid
 486 nitrogen and stored for data collection.

487

488 **Data collection and processing**

489 The data sets were collected at stations I02 and I03 at Diamond Light Source (Oxford,
 490 UK). The diffraction images were processed with AutoPROC⁴⁰ using XDS⁴¹ for
 491 indexing and integration with AIMLESS⁴² and TRUNCATE⁴³ from CCP4 Suite⁴⁴ for
 492 data reduction, scaling and calculation of structure factor amplitudes and intensity
 493 statistics.

494

495 **Structure solution and refinement**

496 MsmCoaB and MsmCoaBC structures were solved by molecular replacement using
 497 PHASER⁴⁵ from the PHENIX software package⁴⁶. For MsmCoaB, the atomic
 498 coordinates of MsmCoaB structure (PDB entry 4QJI) were used as a search model.
 499 Ligand bound structures were solved using our highest resolution MsmCoaB apo form

structure (PDB entry 6TH2). For MsmCoaBC, atomic coordinates of *Arabidopsis*
thaliana CoaC (PDB entry 1MVL)¹⁹ and our highest resolution CoaB structure (PDB
entry 6TH2) were used as search models. Model building was done with Coot⁴⁷ and
refinement was performed in PHENIX⁴⁶. Structure validation was performed using
Coot and PHENIX tools^{46,47}. All figures were prepared using Pymol (The PyMOL
Molecular Graphics System, Version 2.0 Schrödinger, LLC.) and ligand interactions
calculated with Arpeggio⁴⁸.

507

508

509 **High-throughput screening**

Potential inhibitors of CoaBC were assessed at room temperature using a PHERAstar
microplate reader (BMG Labtech). Pyrophosphate produced by CoaB was converted to
two molecules of inorganic phosphate using a pyrophosphatase. Phosphate was then
detected using the BIOMOL® Green reagent (Enzo Life Sciences), which when bound
to phosphate absorbs light at 650 nm. An end-point assay was carried out in clear, flat-
bottom, polystyrene, 384-well plates (Greiner) in an 50 µl reaction volume containing
100 mM TRIS, pH 7.6, 1 mM MgCl₂, 1 mM TCEP, 0.03 U/mL pyrophosphatase, 2 µM
CTP, 40 µM L-cysteine, 30 µM PPA and 30 nM MtbCoaBC. Assays were performed
by adding 25 µL of a 2-times concentrated reaction mixture containing all components
with the exception of the enzymes to all wells, and the reactions started by adding 25 µL
of a 2-times concentrated enzyme mixture. The reaction was carried out for 2 h at room
temperature, before 50 µL of BIOMOL® Green reagent was added and incubated for a
further 20 min prior to reading.

523

524 **Inorganic pyrophosphatase-purine nucleoside phosphorylase PNP-PPIase assay.**

525 The commercially available EnzChek pyrophosphate assay kit (E-6645) (Life
526 Technologies) was used for this assay. The final reaction composition used was 0.03
527 U/mL inorganic pyrophosphatase, 1 U/mL purine nucleoside phosphorylase, 1 mM
528 MgCl₂, 200 μM MESG, 100 mM TRIS pH 7.5, 1 mM TCEP, 32 nM MtbCoaBC, 125
529 μM CTP, 125 μM PPA, 500 μM L-cysteine, and various concentrations of compounds
530 being tested for inhibition, all prepared from DMSO stock solutions (compounds of
531 series one and two) or water (CoA and CoA thioesters). Assays were performed on
532 either a CLARIOstar or PHERAstar microplate reader (BMG Labtech) in 96-well plates
533 (Greiner). A substrate mixture containing the substrates and the inhibitor was pre-
534 incubated at 25 °C for 10 min. An enzyme solution was prepared and separately pre-
535 incubated at 25 °C for 10 min. The reaction was initiated by the addition of the
536 substrates to the solution containing the enzyme to a final volume of 75 μL. Enzymatic
537 activity was monitored by following the absorbance at 360 nm for 30 min (100
538 cycles/20 s each cycle). Assays were performed in triplicates, including a negative
539 control (lacking PPA) and a positive control (lacking inhibitor).

540 Competition assays were performed using the same conditions but with variable
541 substrate concentrations (31.25 μM, 62.5 μM, 125 μM, 250 μM and 500 μM for CTP
542 and PPA, 31.25 μM, 62.5 μM, 93.75 μM 125 μM and 250 μM for L-cysteine).

543

544 ***M. tuberculosis* strains and growth conditions**

545 MIC determination for *M. tuberculosis* H37RvMA was performed as previously
546 described⁴⁹ in the following media: 7H9/ADC/glycerol (4.7 g/L Difco Middlebrook
547 7H9 base, 100mL/L Middlebrook albumin (BSA)-dextrose-catalase (ADC) Difco
548 Middlebrook, 0.2% glycerol and 0.05% Tween-80), 7H9/Cholesterol/Tyloxapol (4.7

549 g/L 7H9 base, 0.81 g/L NaCl, 24 mg/L cholesterol, 5 g/L BSA fraction V and 0.05%
 550 Tyloxapol) and GAST/Fe (0.3 g/L of Bacto Casitone (Difco), 4.0 g/L of dibasic
 551 potassium phosphate, 2.0 g/L of citric acid, 1.0 g/L of L-alanine, 1.2 g/L of magnesium
 552 chloride hexahydrate, 0.6 g/L of potassium sulfate, 2.0 g/L of ammonium chloride, 1.80
 553 ml/L of 10 N sodium hydroxide, 10.0 ml of glycerol 0.05% Tween 80 and 0.05 g of
 554 ferric ammonium citrate adjusted to pH 6.6).

555

556

557

558

559 **Figure Legends**

560

561 **Figure 1: X-ray crystal structure of FMN and CTP bound MsmCoaBC.**

562 (A) Full aspect of the dodecameric CoaBC with CoaC represented in teal and CoaB in
 563 gold. (B) View of a CoaBC dimer with FMN and CTP shown. Each protomer is
 564 coloured differently. The CoaC active site flexible flap is highlighted in blue. (C) In the
 565 left panel, a CoaBC trimer is shown with the CoaC coloured in teal and CoaB in gold.
 566 On the right panel dimerization of two CoaBC trimers is shown with CoaC coloured in
 567 teal or grey for different trimers. Each CoaB forms a dimer with protomers from
 568 different trimers.

569

570 **Figure 2: Detailed view of MsmCoaBC active sites and MsmCoaB dimerisation** 571 **interface.**

572 (A) View of CoaC active site with FMN bound. The active site sits between two
 573 protomers of one trimer (gold and pink) and a third protomer from an adjacent trimer

(green). Hydrogen bonds are depicted in yellow and π -interactions are in blue. (B) Superposition of a CoaB crystal structure in green, with full length CoaBC (teal) showing the active site flaps (brown) of the CoaB and CoaC enzymes. (C) Detailed view of the CTP binding site. Cartoon and residues belonging to each protomer are coloured differently. Hydrogen bonds and π -interactions are coloured as in B. Important waters are represented as red spheres and calcium as a green sphere. Calcium coordination is depicted in purple. (D) CoaB dimerization interface. Each protomer is coloured as in C. (D)

582

583 **Figure 3: Regulation of MtbCoaBC by CoA and CoA thioesters.**

584 (A) Inhibition of MtbCoaBC by CoA, acetyl-CoA, malonyl-CoA and succinyl-CoA. (B) Lineweaver-Burk plots showing the effect of varying the concentration of each substrate in the presence of different concentrations of acetyl-CoA. Error bars represent standard deviation with $n = 3$.

588

589 **Figure 4: Inhibition of MtbCoaBC by compounds 1b and 2b.**

590 (A) Dose response profiles and chemical structure of compounds **1b** and **2b** is shown. (B and C) Lineweaver-Burk plots respectively showing the effect of varying concentrations of compound **1b** and **2b** in the presence of varying concentrations of CTP, PPA and L-cysteine. Error bars represent standard deviation with $n = 3$.

594

595 **Figure 5: MsmCoaB X-ray structure showing the cryptic allosteric site.**

596 CoaB with the cryptic allosteric site closed (A) and opened conformation (B) with compound **1b** (pink) bound. (C) Detailed view of the allosteric site with compound **1b** (yellow) bound. The individual protomers of the CoaB dimer are coloured in green or

pink. Hydrogen bonds are depicted in red, π -interactions are in grey, and hydrophobic interaction in green. Important waters are represented as red spheres and calcium as a green sphere. Calcium coordination is depicted in purple. (D) Gating mechanism of the cryptic allosteric site showing the movement of R207 with the closed conformation in yellow and the open conformation in pink. An *E. coli* structure (PDB code: 1U7Z) with the 4'-phosphopantothenoyl-CMP (purple) intermediate bound is superimposed.

605

Figure 6: Docking of compound 2b into MsmCoaB showing the highest scoring pose. Hydrogen bonds are shown in red. The individual protomers of the CoaB dimer are either coloured in green or pink.

609 Tables

610

Table 1: Inhibition of CoaB domain by CoA, CoA thioesters and the most potent inhibitors from of series one and two. IC₅₀ values determined using the EnzChek pyrophosphate assay are shown. Error represents standard deviation with n = 3.

Compound	IC ₅₀ EnzChek (μM)
CoA	148 ± 11
AcCoA	121 ± 9
MLCoA	49 ± 3
SucCoA	38 ± 2
1b	0.28 ± 0.05
1c	4.6 ± 0.4
2b	0.08 ± 0.01
2c	0.41 ± 0.03
2d	0.54 ± 0.06
2e	3.0 ± 0.2

614

615

Table 2: Inhibitor constants of acetyl-CoA, compound 1b and 2b for the three CoaB substrates. Error represents standard deviation with n = 3.

617

Inhibitor	Variable substrate	K_i (μ M)	Inhibition type [#]
AcCoA	CTP	22.5 ± 1.7	C
	PPA	22.4 ± 1.4	C
	L-cysteine	62.5 ± 3.3	NC
1b	CTP	$*0.222 \pm 0.012$	UC
	PPA	$*0.078 \pm 0.005$	UC
	L-cysteine	$*0.173 \pm 0.007$	UC
2b	CTP	0.093 ± 0.018	Mixed
	PPA	$*0.062 \pm 0.004$	UC
	L-cysteine	$*0.049 \pm 0.003$	UC

* - For uncompetitive inhibitors αK_i product is shown.

- Abbreviations: C – competitive inhibition; NC – non-competitive inhibition; UC – uncompetitive inhibition.

Table 3: Minimum inhibitory concentration (MIC) values of CoaB inhibitors against *M. tuberculosis* H37Rv cultured in different media (μ M).

Compound	7H9/ADC/ Glycerol	7H9/ Cholesterol/ Tyloxapol	GAST/ Fe
1b	>250	>250	125
1c	>250	ND	ND
2b	>250	>250	50
2c	>250	>250	125
2d	>250	>250	ND
2e	>250	ND	ND

*ND – Not determined.

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757

758 **Author contributions**

759 VMendes wrote the manuscript. VMendes designed and performed all the
760 crystallographic experiments with the help of MB, OB and JCW. VMendes and JH
761 designed and performed the kinetic experiments. JH synthesised 4'-
762 phosphopantothenate. PHMT performed docking experiments. DSC performed the
763 native mass spectrometry experiments. SG, TB, SON, SD, JP and CS developed and
764 performed the high-throughput screening. JCE, SLL and HIMB performed the

765 microbiology experiments on *M. tuberculosis* H37Rv. VMendes, JCE, SG, AGC, PCR,
766 KYR, CA, HIMB, CEB, VMizrahi, PGW and TLB managed the project. All authors
767 approved the manuscript.

768

769 **Accession numbers**

770 Coordinates and structure factors related to this work have been deposited in the PDB
771 with accession numbers: **6TGV**, **6TH2** and **6THC**.

Figure 1

A

B

C

Figure 2

Figure 3

A

B

Figure 4

A

B

C

Figure 5

Figure 6