Crystal structure of the TreS–Pep2 complex, initiating α-glucan synthesis in the GlgE pathway of mycobacteria

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ABSTRACT

A growing body of evidence implicates the mycobacterial capsule – the outermost layer of the mycobacterial cell envelope – in modulation of the host immune response and virulence of mycobacteria. Mycobacteria synthesize the dominant capsule component, α(1→4)-linked glucan, via three interconnected, and potentially redundant metabolic pathways. Here, we report the crystal structure of the Mycobacterium smegmatis TreS–Pep2 complex, containing trehalose synthase (TreS) and maltokinase (Pep2), which convert trehalose to maltose-1-phosphate as part of the TreS–Pep2–GlgE pathway. The structure, at 3.6 Å resolution, revealed that a diamond-shaped TreS tetramer forms the core of the complex, and that pairs of Pep2 monomers bind to opposite apices of the tetramer in a 4+4 configuration. However, for the M. smegmatis orthologues, results from isothermal titration calorimetry and analytical ultracentrifugation experiments indicated that the prevalent stoichiometry in solution is 4 TreS + 2 Pep2 protomers. The observed discrepancy between the crystallised complex and the behaviour in the solution state may be explained by the relatively weak affinity of Pep2 for TreS ($K_d$ 3.5 μM at mildly acidic pH) and crystal packing favouring the 4+4 complex. Proximity of the ATP binding site in Pep2 to the complex interface provides a rational basis for rate enhancement of Pep2 upon binding to TreS, but the complex structure appears to rule out substrate channeling between the active sites of TreS and Pep2. Our findings provide a structural model for the trehalose synthase–maltokinase complex in M. smegmatis that offers critical insights into capsule assembly.

Through co-evolution with its host over several millenia Mycobacterium tuberculosis, the organism causing tuberculosis (TB), has developed mechanisms to partially blunt the human immune response. A key role in this finely tuned host-pathogen interaction is played by various components of the complex mycobacterial cell wall, notably lipoarabinomannan (LAM) and phosphatidylinositol-anchored mannan (PIM) (1). Compared to these immunomodulatory cell wall components, the mycobacterial capsule has received relatively little attention. Yet increasing evidence points to a role of the capsule in immune modulation and virulence. For instance, destruction of the capsule by sonication promotes phagocytosis ex vivo (2); capsular polysaccharides bind with high selectivity to DC-SIGN, a key receptor of the innate immune system on dendritic cells (3); deletion of the glgA gene, which encodes a component of the ‘classical’ pathway of α-glucan synthesis, perturbs persistence of bacilli in a mouse
infection model for TB (4); and phagocytosis of non-opsonised bacilli by neutrophils appears to be mediated by capsular α-glucan (5). Finally, double knock-out mutants deleting ADP-glucose pyrophosphorylase-encoding glgC and trehalose synthase-encoding treS led to severe capsule-deficiency and significantly reduced virulence of M. tuberculosis in a mouse infection model (6), underscoring the significance of the capsule in TB infections.

How the capsule is synthesised and assembled is only partially understood. The capsule material is predominantly made of α-glucan (80 – 90%), alongside other key components, including arabinomannan (10 – 20%) and proteins of the ESX-1 secretion system (7). Capsular α-glucan is a polymer of α(1→4)-linked glucose units with α(1→6)-branching, and thus structurally closely related to intracellular glycogen (8,9). To date, three mycobacterial pathways have been implicated in α-glucan (and/or glycogen) synthesis: the ‘classical’ GlgC-GlgA pathway (also present in many other bacteria), the Rv3032 pathway (which leads to methylglucose lipopolysaccharide (10)) and the recently identified TreS-Pep2-GlgE pathway (Fig. 1) (9,11,12). These pathways share either common nodes or are linked by synthetic lethal interactions (9,11,13), conferring an intriguing level of metabolic redundancy. Recent evidence has shown that simultaneous knock-out of the ‘classical’ pathway (GlgC-GlgA) and the GlgE pathway by deleting glgC and treS abolishes capsule formation and significantly reduces virulence, whereas single knock-outs of these two genes partially preserve α-glucan synthesis and have differential effects on α-glucan content in the capsule and the cytosol (6).

In a previous study, we had described the crystal structure of trehalose synthase (TreS) of M. tuberculosis (14), which constitutes the entry point to the 4-step GlgE pathway of α-glucan synthesis (Fig. 1). In the same study, we showed that TreS, which interconverts trehalose and maltose (15), forms a non-covalent complex in vitro and in vivo with maltokinase (Pep2), the second enzyme in the GlgE pathway. Pep2 catalyses phosphate-transfer from ATP to maltose (16), yielding maltose-1-phosphate, which is subsequently polymerised by GlgE into α(1→4)-linked glucan (12). In a fourth reaction step, GlgB introduces α(1→6)-branching of α(1→4)-linked glucan chains (17). Contrary to GlgE or GlgB, neither TreS nor Pep2 are essential enzymes in M. smegmatis, although transposon mutagenesis suggested essentiality of M. tuberculosis Pep2 (18). However, they have been shown to form synthetic lethal interactions with glycosyltransferase Rv3032 (which synthesises linear α-glucan from UDP-glucose), and thus appear to occupy an important metabolic node (11).

The TreS–Pep2 complex of M. tuberculosis contains a tetramer of TreS and four copies of Pep2, with an overall mass of 490 kDa (14). TreS and Pep2 can work independently, but we observed that when M. tuberculosis TreS is added to the M. tuberculosis Pep2-catalysed phosphorylation of maltose, Pep2 activity increased markedly (14). This raised the question by what mechanism TreS is able to affect the catalytic activity of Pep2 and/or whether substrate channeling may be part of the TreS-Pep2 interaction. In order to help address these questions, we undertook crystallisation of the TreS–Pep2 complex. Crystals of the M. tuberculosis complex failed to provide useful diffraction patterns, whereas diffraction data could be obtained with crystals of the orthologues from Mycobacterium smegmatis, diffracting to a resolution of 3.6 Å.

**Results**

**X-ray crystal structure of the M. smegmatis TreS–Pep2 complex.**

The complex of M. smegmatis (Msm) TreS–Pep2 crystallised readily, but crystals of appropriate quality only grew over a narrow pH range (pH 6.7 to 7.0). The structure was phased by molecular replacement, and refined to a resolution of 3.6 Å (Fig. 2, Supporting Table S1). Given the limited resolution, non-crystallographic symmetry (NCS) restraints were applied. The core of the assembly is the previously reported TreS tetramer (14) (Fig. 2A). In the diamond-shaped TreS tetramer, the β-sandwich domains of the TreS subunits are paired up and constitute the ‘top’ (chains A, B) and ‘bottom’ apex (chains C,D) of the diamond (Fig. 2A). In the hetero-octameric TreS–Pep2 complex, pairs of Pep2 monomers straddle across the β-sandwich domains of the TreS tetramer in a clamp-like fashion (Fig. 2B). We refer to this assembly as the 4+4 complex, which measures approximately 175 Å in its long dimension, ~125 Å across and ~90 Å in depth. Pep2 appears to dimerise on the TreS tetramer,
but the contact surface between the Pep2 monomers is small compared to the contact surface with TreS (see below, Fig. 2C), suggesting they bind independently as monomeric copies.

The asymmetric unit (ASU) of the crystal lattice contains two copies of the TreS–Pep2 complex (complexes 1 and 2), which display differences in terms of subunit orientation and level of disorder. For instance, when superimposing the TreS tetramers of complex 1 (chains A – D) and 2 (chains E – H) onto the structure of stand-alone M. smegmatis TreS (PDB entry 3ZO9, (19)), the average displacement (RMSD) of Ca atoms is very small for complex 1 (0.45 Å for 2243 aligned Ca atoms), but considerably larger for complex 2 (RMSD of 3.4 Å, 2175 aligned Ca atoms). The origin of this mismatch are rotations of the TreS protomers relative to each other, evident by altered gaps between the subunits' molecular surfaces when oriented according the alignment described above, and by changes of the distances between the substrate binding sites of up to about 6 Å (Supporting Fig. S1).

The backbone of Pep2 shows various levels of disorder across the eight protomers in the ASU. In complex 1 (Pep2 chains I – L, Fig. 2B), the backbone of Pep2 could be traced over 436 of 441 residues for three subunits (chains I, J and L), but residues 1 to 86 are largely disordered in the fourth subunit (chain K). In ‘complex 2’ (Pep2 chains M – P), only one copy of Pep2 (chain M) could be traced fully, while residues 1–194 were disordered in chain N, O and P.

The structural model of Msm Pep2 in the present complex structure is completely defined by electron density bar two residues of the N-terminal sequence, and 4–5 residues at the C-terminus. Despite the moderate resolution, the density displays sufficient detail to dock the Pep2 amino acid sequence onto the backbone structure, aided in part by the recent structures of M. tuberculosis (Mtb) and M. vanbaalenii (Mvb)Pep2 (20,21) (Supporting Fig. S2). However, side chains were omitted from the model, where corresponding density was missing.

The Pep2 structure shows the prototypical two-lobe architecture of phosphotransferase enzymes of PFAM family PF01636. Characteristic for this family and that of protein kinase domains (PFAM family PF00069) is the α-helical C-terminal lobe (Fig. 3A). This is illustrated by comparison with the closest non-mycobacterial structural neighbour, Bacillus subtilis methylribose kinase (PDB code 2PUL (22), RMSD 3.9 Å, 262 aligned Ca, Fig. 3B), which superimposes with the C-terminal lobe and parts of the N-terminal lobe of Pep2. However, in Pep2 the N-terminal lobe includes a novel β-sheet at the N-terminus (β1 – β3), which appears to be unique for mycobacterial Pep2 enzymes, and is missing in structural neighbours identified by distance matrix alignment (DALI, (23)), including methylribose kinase. In contrast, the 7-stranded β-sheet preceding the C-terminal lobe (β6 to β12) is conserved. We refer to the latter as the canonical β-sheet, although it includes 2 additional, non-conserved strands (β6, β7) at its N-terminus (Fig. 3C).

The unique 3-stranded β-sheet, which is preceded by a 2-turn α-helix, is visible in only a subset of Pep2 protomers (Fig. 3A, 3C), with density lacking between the N-terminus and helix α3 (corresponding to residues 1 to 88) in four of eight Pep2 protomers. Superimposing the Pep2 copies (chains I, J, L and M), which could be fully traced, with respect to residues 198 to 400 of the C-terminal lobe results in a close match for the canonical β-sheet of the N-terminal lobe, but reveals considerable conformational flexibility for the unique β-sheet with a rotation of approximately 28º between the two extreme conformers about the ‘hinge residue’ Leu86 (Fig. 3D). Furthermore, we note that the domain swap seen in the structure of Mtb Pep2 (21) involves strands of the canonical β-sheet (Fig. 3E).

The functional role of the unique β-sheet in Pep2 is not immediately evident from the structure. The nucleotide binding site resides in the N-terminal lobe, whereas maltose binds opposite to nucleotide in a pocket of the C-terminal lobe (21) (Fig. 3A). Superimposing Pep2 with the ATP-bound structure of Myb Pep2 (4WZY, (20)) suggests that residues in the unique region of the N-terminal lobe do not directly contact the nucleotide, but one might expect that perturbations of this part of the structure affect nucleotide binding and potentially activity (see discussion).
**Location of active sites**

Given that TreS and Pep2 catalyse consecutive reaction steps in the GlgE pathway it has been suggested that products of the TreS-catalysed reaction may be channelled directly to the active site of Pep2 (21). However, no such path or channel between the active sites of TreS and Pep2 is apparent in the complex structure. The linear distance from the active site of TreS (chain A) to the nearest maltose binding site of Pep2 (chain J) measures about 53 Å and traverses the C-terminal lobe of Pep2 (Fig. 2D, Supporting Fig. S3). The next nearest maltose binding site (chain I) is 63 Å away and, this time the direct line crosses the hydrophobic core of TreS, again without an obvious opening or tunnel. Within the complex, the active sites of TreS and Pep2 are both open to solvent and do not face each other (Fig. 2D), suggesting that following release from TreS, maltose needs to diffuse through solvent on the way to the active site of Pep2.

**The TreS-Pep2 binding interface**

Pep2 forms intimate contacts with the TreS tetramer, revealing a high level of shape complementarity between the binding partners (Fig. 4A). Complex formation buries about 1200 Å² of solvent accessible surface per monomer. The footprint of a single Pep2 subunit covers surface areas belonging to two TreS protomers, including and extending beyond the C-terminal β-sandwich domain(s) (Fig. 4B). Both lobes of Pep2 contribute to the contact surface, but the C-terminal lobe provides the lion share (Fig. 4C, Supporting Fig. S2). Where parts of the N-terminal lobe are disordered, the C-terminal lobe accounts for all observed contacts. Secondary structure elements contributing to the binding interface are helices α5, α6 and α10 in the C-terminal lobe of Pep2, whereas contacts made by the N-terminal lobe include residues in helix α2, in strand β8 and in the β9–β10 loop (Fig. 4C). In addition, contacts also involve the β12-α5 loop, which links N- and C-terminal lobe. The binding interface is dominated by van der Waals and hydrophobic contacts, corresponding to ~70% of surface area buried in the interface per Pep2 monomer. In addition, 8 hydrogen bonds and 2 salt bridges (TreS-Arg312:Pep2-Asp228, TreS-Arg534:Pep2-Asp218) complement the non-covalent interactions (Supporting Table S2).

The binding interface shares structural elements with the nucleotide-binding site, based on the superposition with the ATP-bound structure of *Mvb* Pep2 (PDB entry 4WZY (20), Supporting Fig. S3). According to this superposition, we predict several residues to make contacts with ATP that are part of (or right next to) the binding interface with TreS. Residues in the maltose binding site (derived from the superposition with maltose-bound *Mtb* Pep2 (4O7P, (21)), make no direct contacts with TreS.

**Stoichiometry and affinity of the M. smegmatis TreS–Pep2 complex in solution**

We had previously investigated the stoichiometry of the complex formed by the *M. tuberculosis* orthologues, and found, by analytical ultracentrifugation in equilibrium mode, that the complex encompasses 4 + 4 subunits (Mr ~ 490,000) (14). However, purified *Mtb* Pep2 eluted from a size exclusion resin as a mix of species (monomers, dimers and higher oligomers) (14), in contrast to the essentially single peak-elution of monomeric *Msm* Pep2 (Supporting Fig. S4). We therefore wished to ascertain, whether the self-association behaviour affected complexation with TreS.

We initially probed assembly of the *Msm* TreS–Pep2 complex by size exclusion chromatography. As was observed previously for the *M. tuberculosis* orthologues, addition of Pep2 to TreS leads to a shift of the TreS tetramer peak to shorter retention times, suggesting complex formation (Supporting Fig. S4). We also noticed that the association of *Msm* TreS and Pep2 was pH-dependent. Between pH 7 and 8, the position of the complex peak moved to longer retention times, while the absorbance signal corresponding to the Pep2 monomer increased (Supporting Fig. S4). In accordance with the stronger association between TreS and Pep2 at mildly acidic pH, the best diffracting crystals were obtained at reservoir pH values between 6.7 and 7.0.

In order to determine the affinity between *Msm* TreS and Pep2, we measured binding by isothermal titration calorimetry (ITC), buffering with phosphate and titrating Pep2 to TreS up to a molar ratio [Pep2]/[TreS] of about 1.5. Setting the initial concentration of TreS at 75 μM, we measured binding isotherms at 25°C at pH 6.5, 7.5 and 8.5 (Table 1 and Fig. 5A). Fitting a single-site binding model, the strongest affinity was measured at pH 6.5 with a Kₘ of 3.5 μM, increasing to 34.8 μM at pH 8.5.
Concentration, cf. Table 2 in Roy et al. (14)). At 200 and 400 mM NaCl (Supporting Table S3), the sedimenting complex is the TreS tetramer binding two copies of Pep2, that is a '4 + 2' stoichiometry. We propose that the TreS tetramer binds 2 copies of Pep2, leading to a monomeric mass of 272,000 Da for the TreS tetramer and 48,800 Da for the Pep2 monomer, and considering that the molar mixture of TreS and Pep2 (3.75, 2.5, and 1.25 µM with respect to monomers) analysed in the same way resulted in a fitted mass of 372,823 Da. Given calculated masses of 272,000 Da for the TreS tetramer and 48,800 Da for the Pep2 monomer, and considering that Pep2 elutes predominantly as a monomeric species from the size exclusion resin (Supporting Fig. S4), the data strongly suggest that the sedimenting complex is the TreS tetramer binding two copies of Pep2, that is a ‘4 + 2’ complex.

**Enzymatic activity of Msm Pep2**

We had observed previously that activity of *Mtb* Pep2 increased markedly in the presence of *Mtb* TreS (14). We tested whether this was also the case for *Msm* Pep2. Given the pH-dependent affinity between *Msm* Pep2 and TreS, we first tested how activity responds to pH, and found that it declined significantly as pH decreased (Fig. 6A, Table 2). Yet even at the lowest pH, *Msm* Pep2 activity was still several orders of magnitudes higher than that of *Mtb* Pep2 in terms of *V*<sub>max</sub> at the same assay conditions (*V*<sub>max</sub> corrected for enzyme concentration, cf. Table 2 in Roy et al. (14)). At pH 6.0, *Msm* Pep2 activity was largely indifferent to adding *Msm* TreS when varying maltose (ATP at 0.3 mM, Fig. 6B). In the converse experiment, adding *Msm* TreS at molar ratios of 1:1 or 4:1 (TreS–Pep2) to *Msm* Pep2 slightly depressed activity at pH 7.5, but had virtually no effect at pH 6.0 (Fig. 6C, 6D). Thus the rate enhancement effect observed for the *Msm* TreS–Pep2 complex does not appear to hold for the *M. smegmatis* orthologues, albeit the latter is more active than the former by several orders of magnitude.

**Is the M. smegmatis complex structure representative of Mtb TreS–Pep2?**

The discrepancy between the TreS–Pep2 complexes of *M. smegmatis* and *M. tuberculosis* in terms of solution stoichiometry and enzymatic behaviour upon complex formation prompted the question whether the present crystal structure is representative of the TreS–Pep2 complex of *M. tuberculosis*. Examining the binding interface, we found that the two salt bridges (TreS-Arg312:Pep2-Asp228, TreS-Arg534:Pep2-Asp218) link residues that are conserved on either side of the interface. In addition, a conserved proline residue in TreS (Pro503), located in the loop between strands β16 and β17, is packing tightly against backbone atoms of helix α5 of Pep2 (Fig. 7A). We reasoned that introducing a bulky side chain at the Pro503 site, or swapping the basic side chains (Arg312, Arg534) for acidic amino acids should be sufficient to destabilise complex formation, given the moderate overall affinity (*K*<sub>d</sub> = 3.5 µM). While *Mtb* TreS-R536E (*Msm* Arg534) did not express well enough, sufficient amounts of protein could be made of *Mtb* TreS-P511W (*Msm* Pro503) and TreS-R320E (*Msm* Arg312). The mutant proteins showed the same elution behaviour on a Superdex 200 size exclusion resin as wild type *Mtb* TreS (Fig. 7B, peaks at 10.2 and 11.7 ml), whereby the peak at 11.7 ml likely represents the TreS tetramer (280 kDa) as it located between the ferritin marker (440 kDa) and the dimer peak for albumin (132 kDa). Thus, this comparison indicates that the mutant TreS proteins maintain the tertiary structure of the wild type enzyme.

To probe Pep2 binding, we incubated *Mtb* TreS (wild type and mutants) with an equimolar amount of *Mtb* Pep2 and analysed the protein mixture by size exclusion chromatography (Fig. 7C – 7E). Only when incubated with wild-type *Mtb* TreS did *Mtb* Pep2 drive a distinct shift of the main TreS...
elution peak at 11.8 ml (Fig. 7C) to 11.4 ml, coinciding with the 440 kDa ferritin marker. This peak contains both proteins (Supporting Fig. S6). In contrast, incubation with either mutant (P511W, R320E, Fig. 7D, 7E) resulted only in a minor shift of the TreS tetramer peak (11.7 ml). The peak representing the Pep2 monomer (15.5 ml), is eliminated in the incubation with wild-type TreS (Fig. 7C), but is rather prominent when Pep2 is incubated with either mutant (Fig 7D, 7E). This differential behaviour clearly indicates that Pep2 binding to the mutant TreS proteins is perturbed by introducing mutations at the TreS–Pep2 interface, thus providing evidence that the Msm TreS–Pep2 complex structure is representative of its counterpart in M. tuberculosis.

Discussion

Non-covalent complexes between enzymes that catalyse successive reaction steps in metabolic pathways provide a means for cells to regulate synthesis of metabolites and to efficiently exploit limited nutritional resource (24). This aspect is especially relevant for M. tuberculosis, an organism that, for the most part, resides sequestered in host macrophages, where it faces a generally nutrient-poor, and in particular carbohydrate-poor environment (25). While the α-glucan capsule is shed readily in culture, previous evidence has implicated α-glucan synthesis in the ability of M. tuberculosis to persist in the host (4) (6), and thus regulatory features of pathways linked to capsule synthesis are likely pertinent for the environmental niche occupied by M. tuberculosis.

Complex formation between TreS and Pep2 is mirrored by TreS-Pep2 fusion enzymes in a sizeable subset of microbial species (96 bacterial, 6 archaeal as listed in (9)). The TreS-Pep2 complex structure is compatible with a fused protein in that the stoichiometry in the crystallised complex is 1:1 and the C-terminal β-sandwich domain of TreS is the docking site for Pep2. The linear distance between the C-terminus of TreS and the nearest ordered N-terminal residue of Pep2 is ~ 40 Å, a distance compatible with sequence insertion in TreS-Pep2 fusion from Pseudomonas aeruginosa, which comprises an additional 16 or 30 residues between the termini of TreS and Pep2 relative to the M. tuberculosis and M. smegmatis orthologues, respectively (Supporting Fig. S7).

The architecture and biochemical behaviour of the TreS-Pep2 complex has parallels to the complex of mycobacterial chorismate mutase (CM) bound to 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) of the shikimate pathway of aromatic amino acid biosynthesis (PDB entry 2W19, (26)). As is the case for TreS, the catalytic domain of DAHP synthase folds as an α/β- or TIM-barrel, and exists as a tetramer in solution. In the hetero-octameric DAHP synthase:CM complex, pairs of CM attach to opposite corners of the DAHP synthase tetramer (Supporting Fig. S8). Importantly, association of CM with DAHP synthase results in strongly amplified mutase activity (26,27). Rate amplification of mutase activity was attributed to a re-arrangement of active site residues upon CM binding to DAHP synthase, leading to molecular environment favouring catalysis (26).

For TreS–Pep2, the level of rate enhancement is modest in M. tuberculosis (cf. Table 2 in ref. (14)) and essentially absent in M. smegmatis, at least under in vitro conditions (Fig. 6C, 6D, Table 2). Given that the individual proteins align with high levels of sequence identity (83% TreS, 66% Pep2), and given the diminished or abrogated binding of Pep2 when the Mtb TreS–Pep2 interface is perturbed (Fig. 7), it is unlikely that complexes from M. tuberculosis and M. smegmatis differ in architecture, even though the domain-swapped dimeric structure of M. tuberculosis Pep2 (21) – possibly a crystallization artifact – confounds the comparison. The domain-swapped Mtb Pep2 dimer is incompatible with the 4 + 4 architecture, as significant steric overlap occurs when superimposing a protomer of the Mtb Pep2 dimer onto, for instance, chain I of Pep2 in the Msm TreS-Pep2 complex (Supporting Fig. S9). Domain swapping is not seen in the stand-alone structure of Mvb Pep2 (20) (61% identity to Mtb Pep2), nor is it occurring in Msm Pep2 in the context of the Msm TreS–Pep2 complex. Also, the size exclusion data clearly show a mixed population of Mtb Pep2 in solution that clearly includes a monomeric species (Fig. 7B). Finally, we lack structures of stand-alone Msm Pep2 and of the Mtb TreS–Pep2 complex.

Notwithstanding the caveats outlined above, the present complex structure gives hints as to how complex formation could affect reaction rates. First, four of the eight Pep2 molecules present in the asymmetric unit display...
a partially disordered N-terminal lobe, which accommodates the ATP binding site. This observation suggests inherent structural flexibility of this region, possibly affecting activity. Indeed, a 70-amino acid N-terminal truncation \((\text{Msm}\ \text{Pep2-\Delta70}, \text{Supporting Fig. S10})\) demonstrates substantially reduced maltokinase activity and affinity to TreS. In size exclusion chromatography, Pep2-\Delta70 showed a diminished shift of the complex peak to shorter retention times, while the dissociation constant, \(K_d\), increased from 3.5 \(\mu\)M to 28.6 \(\mu\)M (Table 1). Notably, the \(\Delta70\)-truncation reduced \(V_{\text{max}}\) 10-fold, from 2513 \((\pm 205)\) to 242 \((\pm 41)\) \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (Supporting Fig. S10).

Second, the complex structure demonstrates that the ATP binding site shares structural elements with the TreS–Pep2 interface (Fig. 4C, Supporting Fig S3). Identifying residues involved in ATP binding by mapping the structure of ATP-bound \(M_{\text{m}}\) Pep2 (4WZY, (20)) onto Pep2 (chain I) of the TreS–Pep2 complex demonstrates a high level of sequence conservation for residues within a 4 Å distance cut-off of ATP (Supporting Fig. S2), and identifies secondary structure elements that are part of the TreS–Pep2 interface and, at the same time, contribute residues to the ATP binding site. These include the \(\beta8-\beta9\) loop, the \(\beta12-\alpha5\) loop, which links N- and C-terminal lobe, and strand \(\beta15\) (Supporting Fig. S3). In contrast, the maltose binding site, identified by alignment with maltose-bound \(M_{\text{h}}\) Pep2 (PDB 4O7P, (21)) is completely conserved for contact residues within a 4 Å distance cut-off (Supporting Figs. S2, S3). Furthermore, the maltose binding site includes far fewer structural elements sharing in the TreS–Pep2 interface and contributing contacts to maltose. Thus, we may attribute the modest rate enhancement seen in the \(M.\ \text{tuberculosis}\) TreS–Pep2 complex to stabilization of the N-terminal lobe of Pep2 when bound to TreS.

We previously provided evidence that complex formation between TreS and Pep2 occurs \textit{in vivo} (14). However, the moderate affinity for binding of Pep2 to TreS \((K_d = 3.5 \mu\text{M})\) suggests that the TreS–Pep2 complex is only transient rather than constitutive (24). This notion is also consistent with the predominant stoichiometry of the \(M_{\text{sm}}\) TreS–Pep2 complex in the solution state (TreS\(_c\):Pep2). It suggests, furthermore, that even as a fusion protein, non-covalent association between the TreS and Pep2 domains may not be constitutive. Such behaviour would resemble endoglucanase EngD from \(C\). \textit{cellulovorans}, where the crystal structure shows non-covalent, intramolecular association between the C-terminal carbohydrate-binding and the catalytic domains, but SAXS analysis demonstrated that the carbohydrate-binding domains do not constitutively associate with the catalytic domains in the solution state (28).

The discrepancy in complex stoichiometry between the crystallised complex and the behaviour in solution is likely explained by crystal lattice contacts, which can influence the thermodynamic equilibrium by (partial) exclusion of solvent and introduction of packing interactions that do not exist in the solution state. Close inspection of the ITC data showed systematic, albeit subtle deviations between the single-site binding model and the data points (Fig. 5A). Introducing a two-site binding model removes the systematic deviation (Fig. 5B) and suggests that the two sites bind with \(K_d\) values of 0.5 \(\mu\)M (site 1) and 18 \(\mu\)M (site 2), respectively (Table 1). The stoichiometry for site 1 remains near 0.5 \((n_1 = 0.45)\), while for the site 2, the stoichiometry is \(n_2 = 0.46\). Thus, overall the stoichiometric ratios of the two sites add up to 0.91, \textit{i.e.} near parity between the complex components, and the difference in magnitude between \(K_d1\) and \(K_d2\) could account for the apparent preponderance of a 4+2 configuration in solution.

Adding parameters to a mathematical model is bound to result in a more accurate description of the observed data. However, the crystal structure elegantly rationalises the binding behaviour observed in the ITC experiment. We propose that the two distinct binding sites (with different affinities) correspond to binding the first and second copy, respectively, of Pep2 to the same apex of the TreS tetramer (Fig. 8). The binding sites for pairs of Pep2 monomers at opposite apices of the TreS tetramer are spatially well separated (Fig. 2A, 2B). Thus, binding of the first copy at one apex (Site 1) does not geometrically constrain binding of the first Pep2 copy at the opposite apex (Site 1’, Fig. 8) and thus binding to Sites 1 and 1’ occurs with the same affinity. However, once an apical position is occupied by a single copy of Pep2, binding of the second copy may be hindered by the first copy and this may lead to an increase of \(K_d\) for the second binding
event. Indeed the conformations of the bound Pep2 copies are not identical. Superimposing Pep2 chains I and J, which are fully ordered and occupy the same apex of the TreS tetramer (Fig. 2B), it emerges that their N-terminal lobes do not match well (Fig. 3D). Forcing the two adjacent copies into an identical conformation results in steric hindrance between their N-terminal lobes. When the N-terminal lobes are truncated (as in Pep2-Δ70), the stoichiometry determined in the ITC experiment converges to \( n = 0.68 \), (Table 1), suggesting that the 4 + 2 configuration is no longer dominant.

In conclusion, we have provided a detailed structural model of the complex formed between trehalose synthase and maltokinase of mycobacteria, with mutagenesis data indicating that this complex structure is a valid representative of the \( \text{Mtb} \) TreS–Pep2 complex.

The structure rationalises rate enhancement of Pep2 induced by binding of Pep2 to the TreS tetramer and, together with the example of the DAHP synthase:CM complex points to complex formation as a mechanism that can contribute to regulating pathway activity in mycobacteria.

**Experimental procedures**

**Cloning of \( M. \text{smegmatis} \) Pep2, TreS.** The DNA sequences of TreS (MSMEG_6515) and Pep2 (MSMEG_6514) were cloned from \( M. \text{smegmatis} \) genomic DNA by polymerase chain reaction (PCR) using the primers listed below:

- **TreS Forward:** GATCGATCATGAGGAGACACGCA
- **TreS Reverse:** GATCGATCAGTCGCCACGATCGGCATCGCCGAC
- **Pep2 Forward:** GATCGATCAGTCGCCACGATCGGCATCGCCGAC
- **Pep2 Reverse:** GATCGATCATGAGGAGACACGCA

The PCR products were ligated into the \( \text{NdeI} \) and \( \text{HindIII} \) sites (underlined) of plasmid vector pET28a (Novagen), and the expressed proteins included an N-terminal His\(_6\)-affinity tag.

**Cloning of \( M. \text{smegmatis} \) Pep2-Δ70.** N-terminally truncated \( \text{Msm} \) Pep2 was cloned from a pET28a vector containing MSMEG_6514 (\( M. \text{smegmatis} \) pep2) using the Phusion High-Fidelity DNA Polymerase kit (New England Biolabs, UK), introducing a STOP codon at the C-terminus. The primers were:

- **Δ70 Forward:** GATCCATATGGTCGCCACGATCGGCATCGCCGAC
- **Δ70 Reverse:** GATCAAGCTTCTACCTCGAAGACGTGC

The purified PCR product (QIAquick extraction) was ligated into the \( \text{NdeI}, \text{HindIII} \) restriction sites of plasmid vector pET28a, yielding a construct encoding N-terminally His\(_6\)-tagged Pep2-Δ70.

**Protein production.** \( E. \text{coli} \) BL21 (DE3) cells were heat-transformed with plasmids, encoding either \( \text{Msm TreS or Pep2} \), and cultured on agar plates (LB/kanamycin 25 \( \mu \text{g/mL} \)). A single colony was used to inoculate 10 mL of LB broth, 1% (w/v) glucose and kanamycin (25 \( \mu \text{g/mL} \)), incubated overnight (37 °C), and propagated to bulk cultures of Terrific Broth media (29) (kanamycin at 50 \( \mu \text{g/mL} \), 37° C 180 rpm). At OD\(_{600}\) = 0.4 – 0.6, the cultures were cooled to 16°C (3 hours), and protein expression induced using 0.6 mM IPTG (TreS) or 0.1 mM IPTG (Pep2), and incubation (12 - 16 h, 16° C, 180 rpm). For Pep2 expression, 10 mM maltose (final conc.) were added to the culture medium prior to induction with IPTG. Cells were harvested (7000 \( g \), 10 min, 4°C), washed with phosphate buffered saline (PBS), resuspended in lysis buffer (30 mL, 25 mM HEPES-NaOH pH 7.6, 10% (v/v) glycerol) including either 0.3 M (TreS) or 1 M (Pep2) NaCl. Pellets were frozen (–80° C), thawed and supplemented with protease inhibitor cocktail (Roche), 1 mM PMSF, 10 mM MgCl\(_2\) and 10 \( \mu \text{g/mL} \) DNase I pellet, as well as maltose (for Pep2 lysate, 100 mM final conc.), followed by passage (4 times) through a French Press (Thermo Spectronic FA-078). The cleared lysate (27,000g, 30 min, 4° C), was diluted 4-fold with buffer A (25 mM HEPES-NaOH pH 7.6, 10% (v/v) glycerol), filtered (0.45 \( \mu \text{m pore size} \)), and loaded on a pre-equilibrated Ni-NTA column (5 mL, GE Healthcare).

The column matrix was washed with buffer A, and with 20 mM, 40 mM and 60 mM imidazole in Buffer A, respectively. The protein was eluted with 500 mM imidazole in buffer A, and fractions analysed by SDS-PAGE. The
eluate was diluted 20-fold with buffer B (20 mM Bis-Tris pH 6.0, 10% (v/v) glycerol – for TreS; 50 mM MES pH 5.8, 10% (v/v) glycerol – for Pep2), filtered (0.45 µm) and applied on a HiTrap Q-column (1 mL, GE Healthcare) pre-equilibrated with 20 mM Bis-Tris pH 6.0, 50 mM NaCl (for TreS) or 50 mM MES pH 5.8, 100 mM NaCl (for Pep2). The column was washed with buffer B supplemented with NaCl (50 to 500 mM, increments of 50 mM). Fractions were analysed by SDS-PAGE and pooled, followed by concentration in Amicon Ultra-4 centrifugal filter units, then loaded on a HiPrep Sephacryl 26/60 S-300HR column (GE Healthcare). Fractions containing protein were concentrated as described before.

The purification of Pep2-Δ70 followed the protocol of wild-type Pep2, except that NaCl was set to 300 mM prior to ion exchange chromatography, rather than 50 mM.

**Crystallisation and structure determination.** Crystals of the TreS–Pep2 complex were grown by vapour diffusion in 96-well plates, using a Mosquito liquid handling system (TTP Labtech) to set up crystallisation drops (100 nL of protein + 100 nL reservoir solution) containing mixes of *M. smegmatis* TreS and Pep2, at molar ratios between 1:1 and 1:2. Well-formed crystals grew over a reservoir of 9 – 10% polyethylene glycol 8000, 4% v/v glycerol, 200 mM MgCl2 and 0.1 M Tris-HCl, pH 6.7 – 7.0. Crystals were immersed briefly in mother liquor supplemented with 15% - 20% (v/v) glycerol, and flash frozen in liquid nitrogen. Diffraction data (Supporting Table S1), were processed using XDS/XSCALE (30). Phases were calculated by molecular replacement (MR) (PHASER, (31)) using PDB entry 3ZO9 (19) as the initial search model. The first of two copies of the TreS tetramer could be placed using a tetrameric search model (pdb code 3ZO9, PHASER, Z-score > 30 after translation search), whereas the second TreS tetramer was placed by searching with a single TreS subunit, then deriving positions of the other 3 monomers by applying NCS operators. The individual protomers were adjusted into clearly visible secondary structure density by real space rigid body fitting (COOT (32)).

An initial model for Pep2 was generated using the HHpred threading server (toolkit.tuebingen.mpg.de/hhpred#, (33)) and the MODELLER software (34), covering residues 100 to 440 of the Pep2 sequence. A first copy of Pep2 was placed by MR (PHASER, Z-score ~ 11), which matched secondary structure density features that had appeared by phasing with TreS alone. Further copies of Pep2 were placed in a cyclic fashion, applying NCS operators, then real space rigid body fitting, refining the combined model in REFMAC5, and reiterating the cycle. After placing 3 copies of Pep2, the resulting σA-weighted 2Fo-Fc density map displayed additional secondary structure elements not covered by the Pep2 model, revealing the location of helix α3 and strands β1 - β3, respectively. Iterative rounds of chain-tracing, model rebuilding and refinement (COOT (32), REFMAC5 (35), PHENIX.REFINE (36)) allowed us to construct a model comprising two TreS tetramers and the backbone of eight Pep2 monomers. In the later stages of building this model, we obtained access to the coordinates of Pep2 from *M. tuberculosis* (PDB entry 4O7O, (21)) and *M. vanbaalenii* (4U94, (20)), which helped to correct the sequence register and to dock side chains onto the backbone structure where justified by density. We applied tight NCS restraints throughout and modelled atomic displacements by refining TLS parameters (one set per protomer) and grouped B factors. Refinement statistics are shown in Supporting Table S1. Structure factors and coordinates for the crystal structure of *M. tuberculosis* TreS–Pep2 are deposited in the PDB under accession code 5JY7

**Size exclusion chromatography.** The elution of equimolar molar mixtures of TreS–Pep2 from a Sephacryl S-300HR resin (320 mL column volume) was assayed at pH 6.0 – 9.0, monitoring UV absorbance at 280 nm with a flow rate of 0.5 mL/min, and loading the column with an initial concentration of 30 µM for each protein. The proteins were buffered in 50 mM Na-phosphate, 300 mM NaCl, 10% (v/v) glycerol. The pH value was set by choosing appropriate ratios of mono- to dibasic Na-phosphate. Fractions (5 mL) were analysed by SDS-PAGE. For the elution of Pep2-Δ70 from the same size exclusion matrix, the protein was in 50 mM MES buffer, pH 6.0, 500 mM NaCl and 10% (v/v) glycerol. The association of Pep2-Δ70 with TreS was analysed in 50 mM Na-phosphate, pH 6.5, 300 mM NaCl, 10% (v/v) glycerol.

**Analytical ultracentrifugation.** Sedimentation equilibrium was carried out in a Beckman
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Optima XL-A analytical ultracentrifuge equipped with absorbance optics. Protein samples were dialyzed overnight in 50 mM Na-phosphate, pH 6.5, 300 mM NaCl and 10% (v/v) glycerol. Equilibrium experiment was performed using 6-channel Epon centerpieces with quartz windows at a rotor temperature of 4 °C for a total duration of 116 hours. Absorbance data were recorded at 280 nm at rotation speeds of 8000, 9000, and 10000. At each rotation speed, the sample was allowed to reach equilibrium during a 24 hours period. Finally, the sample was run at 25,000 rpm for 20 hours. The data were analyzed using SEDPHAT (37). Parameters for solvent density and viscosity and for the partial specific volume of the proteins were calculated using SEDNTERP (sednterp.unh.edu).

Activity assay. In order to monitor phosphorylation of maltose, conversion of ATP to ADP was enzymatically coupled to oxidation of NADH (via pyruvate kinase and lactate dehydrogenase), and the latter monitored fluorimetrically (excitation 340 nm, emission 450 nm). Fluorescence units were converted to concentrations of ADP by an internal calibration curve for NADH. The assays were performed in triplicate in 96-well plates, using a BMG PHERAstar FS microtitre plate reader, and MARS and GraphPad Prism software to record and analyse data, respectively.

Reaction mixtures contained 50 mM Na-phosphate, 300 mM NaCl, 10% (v/v) glycerol, 10 mM MgCl₂, adjusting the pH by mixing mono- and di-basic buffer in appropriate ratios. Enzyme concentrations were 0.45 µM – 1.0 µM for wild-type Pep2 or 1 µM – 2.35 µM for the mutant Pep2-Δ70. When varying ATP, maltose was present at 20 mM, and ATP was at 0.3 mM when varying maltose. The coupling reagents were present at these initial concentrations: 4 mM phosphoenolpyruvate, 2 units pyruvate kinase, 2 units lactate dehydrogenase, and 0.1 mM NADH.

Isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC Microcalorimeter from MicroCal, LLC (Northampton, MA). Both proteins, TreS and Pep2, were dialysed against 50 mM Na-phosphate, pH 6.5 – 8.5 (mixing mono- and dibasic phosphate), 300 mM NaCl and 10% (v/v) glycerol. Prior to the experiment, the buffered protein solutions were degassed while incubated at 2 °C below the experimental temperature (25°C). A total volume of 288 µL of Pep2 (at 500 µM in the syringe) was titrated into 1.4 mL of TreS (75 µM initial concentration with respect to monomer). A total 29 injections (10 µL each time) were made, with a 300 second interval between injections. The data were analyzed using the Origin 7.0 software package (Microcal, Northampton, MA). The experimental data were fitted to models describing either a single or two binding sites.

Site-directed mutants of Mtb TreS were generated by de novo gene synthesis (Genescript) and the resulting DNA sequences (codon-optimised for expression in E. coli) were subcloned into pET28a plasmids containing a kanamycin resistance cassette. Plasmids were verified by sequencing, and heat shock transformed into E. coli BL21(DE3) cells, and transformants cultured on LB agar plates selecting for kanamycin resistance (50 µg/mL kanamycin, 37°C, overnight). Proteins were purified from cell extracts as described under ‘Protein production’, but replacing the ion exchange with a size exclusion column (Superdex 200 Increase 10/300 GL, GE Healthcare Life Sciences). Fractions of the Ni-NTA eluates containing the His-tagged proteins were pooled, concentrated to a volume of ≤ 1 mL (Amicon Ultra, Mr > 10 kDa) and applied to the Superdex column, which was pre-equilibrated in 50 mM NaPO₄, pH 6.5, 100 mM NaCl and 10% v/v glycerol, with buffer exchange occurring through the size exclusion step.

Complex formation between Mtb Pep2 and Mtb TreS (wild type, or mutants P511W, R320E) was probed by size exclusion chromatography (Superdex 200 Increase 10/300 GL, bed volume 24 ml), monitoring UV absorbance at 280 nm. Prior to loading the column, equimolar amounts of Mtb Pep2 and Mtb TreS (wild type or mutants) were mixed, incubated on ice for 30 minutes, then loaded on the column and eluted with a flow rate of 0.5 mL/min for a total elution volume of 30 ml. Elutions from the column were calibrated using bovine albumin (M, 66 kDa) and ferritin (M, 440 kDa).
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Author contributions: AAK, LJA, GSB and KF designed study; AAK, RR, CG, KK, TRP generated reagents and collected data; AAK, TRP, LJA, and KF analysed data; AAK, KK, LJA and KF generated figures; AAK, GSB, KF wrote and edited the manuscript, which was reviewed by all authors.
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**FOOTNOTES**

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The abbreviations used are: AUC, analytical ultracentrifugation; ITC, isothermal titration calorimetry; LAM, lipoarabinomannan; NCS, non-crystallographic symmetry; PIM, phosphatidylinositolmannose; TB, tuberculosis;
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Tables
Table 1. Isothermal titration calorimetry (ITC) data of the TreS-Pep2 interaction. The sample chamber contained 75 µM *M. smegmatis* TreS at the start of the titration, and Pep2 or Pep2-Δ70 were titrated to TreS up to a molar ratio of about 1.5 of [Pep2]/[TreS].

|             | Pep2 (wild-type) | Pep2-Δ70 |
|-------------|------------------|----------|
| pH          | 6.5              | 7.5      | 8.5      | 6.5      |
| T (K)       | 298              | 298      | 298      | 298      |
| $K_a$ (10^5 M^-1) | 2.87 ±0.26     | 1.60 ±0.12 | 0.287 ±0.03 | 0.35 ±0.08 |
| $K_d$ (µM)  | 3.48             | 6.25     | 34.8     | 26.8     |
| $\Delta G^o$ (kJ/mol) | -31.1           | -29.7    | -25.4    | -25.9    |
| $\Delta H^o$ (J/mol) | -131,000        | -78,400  | -93,200  | -74,900  |
| $T\Delta S^o$ (J/mol) | -99,400         | -48,700  | -67,600  | -49,000  |
| n           | 0.574            | 0.459    | 0.22     | 0.681    |
|             | ±0.007           | ±0.008   | ±0.04    | ±0.004   |
| $K_{a1}$ (10^5 M^-1) | 19.7±3.2        |
| $K_{a2}$ (10^5 M^-1) | 0.55±0.08       |
| $K_{d1}, K_{d2}$ (µM) | 0.5, 18         |
| $n_1, n_2$  | 0.445±0.004,    | 0.457±0.055 |

Single-site binding model

Two-site binding model
Table 2. Enzymatic activity of *M. smegmatis* Pep2. Activity was probed through a coupled assay, spectroscopically monitoring NADH depletion (see Experimental Procedures). Maltose was at 20 mM when titrating ATP, and ATP was at 0.3 mM when titrating maltose.

| [TreS]/[Pep2] as indicated | ATP titration, [TreS] = 0 | Maltose titration, pH = 6.0, [TreS]/[Pep2] as indicated |
|----------------------------|--------------------------|--------------------------------------------------|
| pH 7.5                     | pH 7.0                   | pH 6.5                                           |
| *V*<sub>max</sub> (µmol min<sup>-1</sup> µg<sup>-1</sup>) | 1901                     | 1993                                              |
| *K*<sub>M</sub> (mM)        | 0.076                    | 0.099                                             |
| Standard errors            |                          |                                                  |
| *V*<sub>max</sub>          | 88                       | 43                                               |
| *K*<sub>M</sub>            | 0.007                    | 0.004                                            |

95% Confidence Intervals

| [TreS]/[Pep2] as indicated | ATP titration, pH = 7.5 | ATP titration, pH = 6.0 |
|----------------------------|-------------------------|-------------------------|
| 0:1                        | 1:1                     | 4:1                     |
| *V*<sub>max</sub> (µmol min<sup>-1</sup> µg<sup>-1</sup>) | 1901                     | 1716                   |
| *K*<sub>M</sub> (mM)        | 0.076                    | 0.091                  |
| Standard errors            |                          |                        |
| *V*<sub>max</sub>          | 88                       | 71                    |
| *K*<sub>M</sub>            | 0.007                    | 0.007                 |

95% Confidence Intervals

Goodness of fit: R<sup>2</sup>
Figures

Figure 1. Schematic diagram of the GlgE-pathway of mycobacterial α-glucan synthesis. Conversion of trehalose to maltose-1-phosphate proceeds through isomerisation (TreS) and phosphorylation (Pep2) of the disaccharide (generated using ChemBioDraw).

Crystal structure of the TreS–Pep2 complex

Trehalose  Maltose  Maltose-1-phosphate

\[
\begin{align*}
\text{Trehalose} & \quad \text{Maltose} & \quad \text{Maltose-1-phosphate} \\
\text{TreS} & \quad \text{Pep2} & \quad \text{GlgE}
\end{align*}
\]

\[
\text{ Conversion of trehalose to maltose-1-phosphate proceeds through isomerisation (TreS) and phosphorylation (Pep2) of the disaccharide (generated using ChemBioDraw).}
\]
Figure 2. Architecture of the *M. smegmatis* TreS–Pep2 complex. (A) View of the TreS tetramer, illustrating juxtaposition of the C-terminal β-sandwich domains (hues of magenta) at the apices of the tetramer. The tetramer shown is from the first of two complex copies in the asymmetric unit (complex 1) with chain identifiers A – D. (B) Assembly of the TreS–Pep2 complex in the orientation of panel A, with TreS and Pep2 subunits shown in blue and green ribbons, respectively. Spheres indicate the location of the active sites, as derived from the superposition with substrate-bound homologous structures. Capital letters designate the chain identifiers in complex 1. (C) Top view of the assembly shown in panel B, with Pep2 subunits rendered as molecular surfaces. The rotation relative to panel B is indicated. (D) Illustration of the relative spatial positions of the active sites of TreS and Pep2. The line in magenta shows the linear distance (53 Å) between the disaccharide binding sites, and the openings of the active sites to solvent are marked.
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Figure 3. Structure of *M. smegmatis* Pep2 and topology of its N-terminal lobe. (A) Ribbon diagram of the *M. smegmatis* Pep2 (MsPep2) monomer. The conserved and unique regions in the N-terminal lobe are shown in hues of blue, matching the colour scheme of the topology diagram in panel C. Spheres in grey indicate the binding sites of nucleotide and maltose, as derived from secondary structure-matched superposition with GDP-bound aminoglycoside 2”-phosphotransferase IIIa (PDB entry 3TDW, (38)) and maltose-bound *M. tuberculosis* Pep2 (4O7P, (21)), respectively. (B) Ribbon diagram of *Bacillus subtilis* methylribose kinase (2PUL, (22)), the closest structural neighbour of Pep2 according to DALI (23) (RMSD 3.9 Å for 262 aligned Cα positions, 12% identity). (C) Topology diagram of the N-terminal lobe of MsPep2. (D) Superposition of MsPep2 chains I, J, L and M with respect to residues 198 – 400. (E) Superposition of MsPep2 with *M. tuberculosis* Pep2 (MtPep2) (4O7O, (21), grey ribbon). Secondary structure labels referring to MtPep2 are preceded by the prefix ‘Mtb’. The Cα-traces deviate following strand β7, where the backbone of MsPep2 (magenta) traverses to the other end of the canonical β-sheet (β8), while the backbone of MtPep2 (yellow) extends to the adjacent, NCS-related copy of Pep2 in a domain swap-mediated dimerization.
Figure 4. Contact surfaces between TreS and Pep2. (A) Side view of the TreS–Pep2 interface for chain I of MsPep2. The surface of TreS is coloured grey and in hues of magenta (indicating the C-terminal β-sandwich domain of TreS). MsPep2 is coloured in hues of blue (N-terminal lobe) and orange (C-terminal lobe). Contact surfaces between TreS and Pep2 are coloured green (on TreS) and yellow (on Pep2) according to burial of solvent exposed surface (calculated using PISA (39)). (B) Area (green) on the molecular surface of TreS contacted by chain I of MsPep2 as seen from the position of the Pep2 subunit. The dashed line indicates the boundary between the C-terminal β-sandwich and the TIM-barrel domains of TreS. (C) Area on Pep2 (chain I) contacted by TreS, as seen from the viewpoint of the latter. Areas in yellow and green contact TreS chains B and A, respectively.
Figure 5. Probing binding between *M. smegmatis* TreS and Pep2 by isothermal titration calorimetry and sedimentation equilibrium analysis of the *M. smegmatis* TreS–Pep2 complex. (A, B) Proteins were in Na-phosphate buffer at pH values of 6.5, 7.5 and 8.5. The starting concentration of TreS in the reaction chamber was 75 μM, and Pep2 was titrated up to nominal molar ratio of ~1.5. (A) Trace of the injections at pH 6.5 and plot of integrated peak areas vs concentration ratio [Pep2]/[TreS], with the fit representing a single-site binding model. (B) Fit of a two-site binding model to the data in panel A. (C) A 1:1 molar mixture of *M. smegmatis* TreS and Pep2 was analysed at three different protein concentrations ([TreS] = 3.75, 2.5 and 1.25 μM) and three rotation speeds (8,000, 9,000 and 10,000 rpm). Data points are shown as open symbols, and solid lines represent the best fit (top panel) and residuals (lower panel), respectively. The fitted mass was 372,823 Da, compared to a calculated mass of 370,407 Da for a complex of 4 TreS + 2 Pep2. Data shown illustrate the fit for the highest protein concentration (see also Supporting Fig. S5).
Figure 6. Activity of *M. smegmatis* Pep2 as a function of pH and in presence/absence of *M. smegmatis* TreS. Activity of Pep2 was analysed, coupling generation of ADP to depletion of NADH (see Methods). (A) pH dependence of activity in presence of 20 mM maltose. (B) Pep2 activity at pH 6.0 as a function of maltose concentration (0.3 mM ATP) and adding TreS at molar ratios as indicated; (C, D) Probing the effect of *M. smegmatis* TreS on Pep2 activity at pH 7.5 and 6.0, respectively, with maltose at 20 mM. Data in panels A to D were fitted to the Michaelis-Menten equation \( v_i = \frac{V_{max} [S]}{K_M + [S]} \). Error bars are omitted where they appear smaller than the corresponding data point marker.
Figure 7. Probing *M. tuberculosis* TreS–Pep2 complex formation with amino acid substitutions at the binding interface. (A) Location of the substitution sites *Msm* Pro303 (*Mtb* Pro311) and *Msm* Arg312 (*Mtb* Arg320), with a close-up view on the right. Pep2 is shown with a translucent molecular surface (blue and orange ribbon), while TreS is shown as a ribbon only (grey, purple). Residue numbers refer to the *Msm* sequences, with corresponding *Mtb* residue numbers in parentheses. Selected secondary structure elements are labelled, with letters T, P indicating TreS and Pep2, respectively. (B) Size exclusion profiles of *Mtb* TreS (wild type, P511W, R320E) and of *Mtb* Pep2 in the absence of their complex partners. Void volume and position of markers ferritin (11.4 ml) and albumin (14.8 ml, 13 ml) are indicated. (C – E) Size exclusion profiles of *Mtb* TreS (wild type, P511W and R320E) in the presence of an equimolar amount of *Mtb* Pep2 superimposed over the elution traces of free TreS of panel B. For the ease of comparing separate runs, the absorbance signals were scaled such that the maximal absorbance is indicated as 1.0. To facilitate a direct comparison between the elution behaviour of TreS–Pep2 complex (orange) to that of the constituent proteins, the elution traces of Pep2 (grey) and of the relevant forms of TreS (wild-type or mutant) of panel B appear again in panels C to E (in blue).
Figure 8. Illustration of the 2-site binding model for binding of 4 copies of Pep2 to the TreS tetramer. Binding of the first copy of Pep2 at an unoccupied apical binding site (Site 1) is governed by $K_{d1}$ and does not influence affinity for binding of the first copy at the opposite apex (Site 1’). Hence Site 1 and Site 1’ have identical dissociation constants. Binding of the second copy of Pep2 may encounter steric constraints imposed by the previously bound copy, and therefore the affinity for binding the second copy, $K_{d2}$ at Site 2 (or Site 2’) is different.
Crystal structure of the TreS-Pep2 complex, initiating α-glucan synthesis in the GlgE pathway of mycobacteria
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