Homotypic dimerization of a maltose kinase for molecular scaffolding

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Mycobacterium tuberculosis (MtB) uses maltose-1-phosphate to synthesize α-glucans that make up the major component of its outer capsule layer. Maltose kinase (MaK) catalyzes phosphorylation of maltose. The molecular basis for this phosphorylation is currently not understood. Here, we describe the first crystal structure of MtBMaK refined to 2.4 Å resolution. The bi-modal architecture of MtBMaK reveals a remarkably unique N-lobe. An extended sheet protrudes into ligand binding pocket of an adjacent monomer and contributes residues critical for kinase activity. Structure of the complex of MtBMaK bound with maltose reveals that maltose binds in a shallow cavity of the C-lobe. Structural constraints permit phosphorylation of α-maltose only. Surprisingly, instead of a Gly-rich loop, MtBMaK employs ‘EQS’ loop to tether ATP. Notably, this loop is conserved across all MaK homologues. Structures of MtBMaK presented here unveil features that are markedly different from other kinases and support the scaffolding role proposed for this kinase.

Trehalose is a non-reducing disaccharide found in bacteria, archaea, fungi, plants and some invertebrates1,2. Surprisingly, mammalian cellular systems show a complete lack of trehalose3. In mycobacteria, trehalose serves as a component of cell wall glycolipids, and plays an important role in cellular response to stress4. Notably, trehalose is constitutively present in the cytoplasm of mycobacteria and is constantly subject to turnover; exemplifying the importance of trehalose in mycobacterial metabolism1. A key conversion of trehalose to α-glucans for the synthesis of the outer capsule layer and possibly methylglucose lipopolysaccharides contributes to the pathogenicity of Mycobacterium tuberculosis (MtB)5–7. At least three different pathways are known to exist for the synthesis of α-glucans. These include the GlgC-GlgA pathway8, the (1→4) glucosyltransferase (Rv3032) pathway9 and the TreS-MaK-GlgE-GlgB pathway10. These pathways have been shown to compensate each other for malfunctions in the pathways that stall α-glucan synthesis. For example, ΔGlgE-ΔTreS mutants of mycobacteria with a deficient TreS-MaK-GlgE-GlgB pathway are viable11. When an additional loss-of-function mutation of Rv3032 was introduced in these mutants, they lost viability, highlighting the redundancy in pathways for synthesis of α-glucans12.

Maltose kinase functions in the TreS-MaK-GlgE-GlgB pathway that has been shown to synthesize α-glucans8. In this pathway, trehalose is first isomerized to maltose by trehalose synthase (TreS). This conversion is reversible13. Next, maltose is phosphorylated to maltose-1-phosphate by maltose kinase (MaK) by expending a molecule of ATP. The phospho-activated disaccharide is a substrate for glucosyltransferase E (GlgE). GlgE uses maltose-1-phosphate to elongate α(1→4) linked glucan chains. The last enzyme of this pathway, glucosyltransferase B (GlgB), mediates α(1→6)-branching of the glucan chain14 (Supplementary Fig. S1). Interestingly, inactivation of GlgE leads to accumulation of maltose-1-phosphate, the product of MaK, during growth in presence of trehalose. The accumulation of maltose-1-phosphate has been shown to be the cause of rapid death of MtB in vitro and in mice15.

In vitro viability studies on MtB subjected to transposon mutagenesis have helped identify mak as an essential gene for the growth of MtB12,13. The product of MaK-mediated catalysis, maltose-1-phosphate, is used for synthesis of α-glucans that make up almost 80% of the components of the outer capsule of MtB14. However, unlike MtB, micro-organisms like Actinoplanes missouriensis and Streptomyces coelicolor that express MaK homologues do not produce outer capsule layer. In addition, MaK is constitutively expressed in Actinoplanes missouriensis15. These observations suggest the possibility of additional roles for maltose-1-phosphate other than synthesis of capsular α-glucans. Biophysical characterization of MtBMaK using size exclusion chromatography and sedimentation velocity experiments suggests that MtBMaK exists as a mixture of monomers, trimers and tetramers in solution16. Notably, MtB forms a complex with TreS in vitro and in vivo17. This is not surprising partly...
due to the fact that the mak gene is usually linked with the treS gene and in some micro-organisms like Psuedomonas entamophila and Rubrobacter xylophilus the two genes are fused into a single gene\(^5\).

Four units of MtbMaK associate with a tetramer of TreS to form a hetero-octameric complex\(^7\). More importantly, the formation of the complex enhances the activity of MtbMaK by 3-folds\(^7\). Although the crystal structure of the tetramer of TreS is known\(^7,17,18\), the molecular basis for the enhancement of activity of MaK by TreS is currently not understood. In addition, the structural basis for conversion of maltose to maltose-1-phosphate is not known.

Here, we describe the crystal structures of the apo- and maltose-bound MtbMaK. Unexpectedly, the structures reveal a unique N-lobe that has not been observed before in structures of other kinases. MaK uses homotypic dimerization to assemble the active sites for catalysis. Maltose binds in a spherical cavity of the C-lobe with the O1’ oxygen atom oriented towards the catalytic D322. Instead of a glycine-rich loop, MaK uses a LIEQSNXS\(^4\) motif conserved across MaK homologues to catalyse the phosphorylation of maltose. These structural variations and a putative role for the signal independent homotypic dimerization of MaK in scaffolding are discussed.

**Results**

**Structure of MtbMaK reveals a unique N-lobe.** Structure of the apo-enzyme was solved by single-wavelength anomalous diffraction (SAD) method using crystals derivatized with HgCl\(_2\) (Table 1). The final model consists of amino acids 5–455, 1 molecule of maltose, 2 molecules of Bis-Tris, and 1 SO\(_4^{2-}\) ion refined to 2.9 Å resolution with an R factor of 0.239 and a free R value of 0.281.

The overall structure of MtbMaK can be divided into an N-lobe primarily composed of \(\beta\) strands and a C-lobe made up of mostly \(\alpha\)-helices (Fig. 1a). Unlike typical kinases, the N-lobe of MaK contains not one, but two \(\beta\)-sheets. Each sheet encloses an \(\alpha\) helix and a short 3\(_{10}\) helix. The sheet proximal to the N-terminus of MtbMaK is highly twisted and is formed by three long (\(\beta_1\)–\(\beta_3\)) and two short (\(\beta_4\) and \(\beta_5\)) strands running anti-parallel to each other. In contrast, the second sheet is made of seven anti-parallel strands (\(\beta_6\)–\(\beta_7\), \(\beta_9\)–\(\beta_{11}\), \(\beta_8\)–\(\beta_9\)), that includes two strands (\(\beta_8\)–\(\beta_9\)) contributed by an adjacent monomer. In exchange, strands \(\beta_8\) and \(\beta_9\) of this sheet protrude out of the protein, insert themselves into the adjacent monomer and make up two strands of the corresponding sheet in the adjacent monomer. This results in the formation of an unusual homotypic dimer of MtbMaK (Fig. 1b). The unique arrangement of the structural elements of the N-lobe of MtbMaK constitutes a new fold. In addition, the signal independent homotypic dimerization of MtbMaK involving mutual exchange of structural elements from the N-lobe, has not been observed before for any kinases. In contrast to the N-lobe that is very different from the N-lobes of most kinases, the overall structure of the C-lobe of MtbMaK resembles the C-lobes found in most kinases\(^19\).

**Comparison with homologous kinases.** 3D structure alignment-based similarity search using Dali\(^20\) retrieved methylthioribose (MTR) kinase (PDB code 2PUN; 13% sequence identity, r.m.s. deviation of 3.9 Å for 249 overlapping C\(_\alpha\) atoms; Fig. 1c)\(^21\) and choline kinase (PDB code 1NW1; 16% sequence identity, r.m.s. deviation of 3.6 Å for 228 overlapping C\(_\alpha\) atoms)\(^22\) as structural matches. Other significant hits included aminoglycoside phosphotransferases

### Table 1 | Data collection and refinement statistics

| Dataset | Hg derivative (peak) | MtbMaK (native) | MtbMaK-maltose |
|---------|----------------------|----------------|---------------|
| Space group | P6\(_1\)\(_2\)2\(_1\) | P6\(_1\)\(_2\)2\(_1\) | P6\(_1\)\(_2\)2\(_1\) |
| Unit-cell parameters | | | |
| \(a, b, c\) [Å] | 97.3, 97.3, 464.0 | 96.6, 96.6, 459.7 | 96.7, 96.7, 461.3 |
| \(\alpha, \beta, \gamma\) [°] | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| Wavelength [Å] | 1.0052 | 1.0000 | 1.0000 |
| Resolution [Å] | 50.00–3.20 (3.31–3.20) | 50.00–2.40 (2.49–2.40) | 50.00–2.90 (3.00–2.90) |
| Total observation | 282,429 | 910,859 | 195,018 |
| Unique reflections | 22,780 | 51,256 | 29,699 |
| Data completeness (%) | 99.4 (99.1) | 100.0 (100.0) | 99.7 (99.8) |
| \(R_{merge}^{b}\) | 0.138 (0.753) | 0.082 (0.548) | 0.062 (0.549) |
| \(I/\sigma(I)\) | 16.2 (3.6) | 35.3 (4.2) | 25.5 (3.3) |
| Redundancy | 12.4 (12.5) | 17.6 (9.2) | 6.6 (6.7) |
| Refinement | | | |
| Resolution [Å] | 38.31–2.40 | 44.60–2.90 |
| Reflections used | 50,237 | 28,826 |
| \(R_{work}/R_{free}^{c}\) | 0.207/0.244 | 0.239/0.281 |
| No. of non-H atoms | 3,349/3,490 | 3,349/3,490 |
| Chain A/B | 159 | 56 |
| Ligands and Water | | |
| Average B factor [Å\(^2\)] | 67.4/63.1 | 116.0/116.9 |
| Chain A/B | 50.5 | 113.2 |
| Ligands and Water | | |
| R.m.s. deviation | | |
| Bond lengths [Å] | 0.017 | 0.021 |
| Bond angles [°] | 1.249 | 1.699 |
| Ramachandran plot (%) | | |
| Favored | 96.9 | 96.8 |
| Allowed | 3.1 | 3.2 |
| Outliers | 0 | 0 |

\(^{a}\)Values in parentheses are for the highest resolution shell.

\(^{b}\)\(R_{merge} = \frac{\sum_h |I_h| - \langle |I_h| \rangle}{\sum_h |I_h|} \langle |I_h| \rangle\), where \(\langle |I_h| \rangle\) is the mean intensity of the observations of \(I_h\) of reflection h.

\(^{c}\)\(R_{work} = \frac{\sum_h |F_h| - \langle |F_h| \rangle}{\sum_h |F_h|}\), where \(F_h\) and \(F_o\) are the observed and calculated structure factor amplitudes of reflection h. \(R_{free}\) is mathematically equivalent to \(R_{work}\), but was measured over 5% of the data.
and many eukaryotic protein kinases such as cell division protein kinase, casein kinase, proto-oncogene serine/threonine-protein kinase and cAMP-dependent protein kinase (PKA).

Since protein kinases have been characterized extensively and also because MtbMaK shares similarities with eukaryotic protein kinases, key features of MtbMaK are discussed in context with terminologies used for typical protein kinases. Comparisons with prokaryotic structural homologues like MTR and choline kinases bring out remarkable differences; illustrating MtbMaK has evolved specific features for its function. A common conserved feature found in most kinases is the presence of a glycine-rich loop (GxGxxG) between strands β1 and β2. Variations in composition of this loop that is involved in positioning of phosphate groups of ATP have been observed previously. The structure of MtbMaK unveils a new variation, an EQS\textsuperscript{142} sequence, at a structurally equivalent position (Fig. 1d). Interestingly, MTR kinase has a GxGxN motif for binding ATP\textsuperscript{23} and choline kinase shows the presence of a di-glycine within the β1-β2 loop\textsuperscript{22}. A lysine from β3 strand of the N-lobe usually makes contact with α and β phosphates of ATP. K157 from strand β9 of MtbMaK and K61 from strand β3 of MTR kinase are located in equivalent positions. In choline kinase, R111 replaces the lysine at this position. Instead of a glutamate, D169 from the αC helix of MtbMaK makes a salt bridge with K157, analogous to the salt bridge for the formation of an activated "αC-in" conformation of protein kinases. In case of MTR kinase, E84 located on αC helix makes a typical salt bridge with K61. The corresponding glutamate E125 is dis-ordered in the structure of choline kinase. Absence of salt bridge signifies an inactive conformation. This is in agreement with the fact that choline kinase was crystallized in presence of Ca\textsuperscript{2+} ions that are known to inactivate the kinase\textsuperscript{12}. The active site of kinases usually contain two aspartate residues; one each located on the catalytic and activation loops. D322 from the 320HGD322 motif of MtbMaK is within hydrogen bonding distance of O1\textsuperscript{9} hydroxyl oxygen of maltose and can function as the key catalytic residue. D322 from the 320HGD322 motif of MtbMaK is within hydrogen bonding distance of O1\textsuperscript{9} hydroxyl oxygen of the substrate. Intriguingly, choline kinase has a...
HND motif with D255 occupying a catalytically equivalent position. The second aspartate of the active site is part of the activation loop that positions the Mg\(^{2+}\) ions for catalysis. D339 of *Mtb*MaK from the \(^{339}\)DFE\(^{341}\) motif, D250 of MTR kinase and D301 of choline kinase located within a DXE motif are well positioned to perform this role. Thus, although *Mtb*MaK shares many catalytic features with other kinases, key differences like presence of the ‘EQS’ loop, which is surprisingly widely distributed in MaK homologues, suggests function-specific alterations of the kinase.

*Mtb*MaK exhibits a unique mode of dimerization. Analysis of the asymmetric unit of the crystallized *Mtb*MaK revealed the presence of two molecules of the protein. These two molecules of *Mtb*MaK form a dimer via homotypic interactions that involve backbone atoms as well as side chains. Strands \(\beta 8\) and \(\beta 9\) from one monomer protrude out and insert into the active site of another monomer (Fig. 2a). Amino acids N145-F159 making up strand \(\beta 9\) of the monomers within a dimer run anti-parallel with the backbone atoms from both the strands interacting along the entire length of the strands (Fig. 2b). Additional intermolecular interactions are observed between side chains of residues from the \(^{142}\)EQS\(^{144}\) loop and the loop connecting helix \(\alpha 1\) with strand \(\beta 1\). Interestingly, intermolecular interactions between the \(^{142}\)EQS\(^{144}\) loop and the “activation loop” are also observed. Further, the loop connecting strand \(\beta 7\) with \(\beta 8\) interacts with residues connecting strands \(\beta 3\) with \(\beta 4\) and helix \(\alpha 2\) (Fig. 2c). Details of amino acids involved in inter-molecular interactions are listed in Table S1. PISA\(^\text{a}\) analysis revealed that dimerization of *Mtb*MaK buries 2,575 Å\(^2\) of solvent accessible surface area. Intriguingly, structural matches like MTR kinase, choline kinase and aminoglycoside phosphotransferase type IIIa (APH IIIa) retrieved by Dali analysis for *Mtb*MaK, are also known to form dimers\(^{17,22,24}\). However, dimerization of these kinases involves participation of C-lobe of one of the monomers. On the other hand, crystal structure of the kinase domain of a transmembrane Thr/Ser kinase, PknB (PDB code 1MRU)\(^\text{25}\), does show the N-lobe forming intermolecular interactions. But this mode of dimerization mediated by the N-lobe is very different from that of MaK. While dimerization involves surface residues in PknB, structural elements are mutually exchanged between the N-lobes of two monomers of MaK. As a result, dimerization of *Mtb*MaK buries a much larger solvent accessible surface area (2,575 Å\(^2\)) when compared to that buried during dimerization of PknB (1,045 Å\(^2\)). Dimerization involving mutual exchange of structural elements has been seen previously in human ChPK2 (Supplementary Fig. S2)\(^\text{26}\). However, ChPK2 dimerization involves the transactivation loop (T-loop) of the C-lobe and not the N-lobe. Thus, the mode of dimerization of *Mtb*MaK is unique. Extensive inter-subunit interactions result in the formation of a tight dimer of *Mtb*MaK. This dimerization of *Mtb*MaK is signal independent and involves homotypic interactions.

Maltose-binding site of *Mtb*MaK. To gain insights into the nature of maltose binding site, we solved the structure of *Mtb*MaK in complex with maltose. As observed for the unliganded enzyme, *Mtb*MaK bound with maltose crystallized as a dimer. Electron density for maltose in Chain B was unambiguous (Fig. 3a). Maltose binding site, we solved the structure of *Mtb*MaK in complex with maltose. As observed for the unliganded enzyme, *Mtb*MaK bound with maltose crystallized as a dimer. Electron density for maltose in Chain B was unambiguous (Fig. 3a). Maltose binding site, we solved the structure of *Mtb*MaK in complex with maltose. As observed for the unliganded enzyme, *Mtb*MaK bound with maltose crystallized as a dimer. Overall, crystal structure of the kinase domain of a transmembrane Thr/Ser kinase, PknB (PDB code 1MRU)\(^\text{25}\), does show the N-lobe forming intermolecular interactions. But this mode of dimerization mediated by the N-lobe is very different from that of MaK. While dimerization involves surface residues in PknB, structural elements are mutually exchanged between the N-lobes of two monomers of MaK. As a result, dimerization of *Mtb*MaK buries a much larger solvent accessible surface area (2,575 Å\(^2\)) when compared to that buried during dimerization of PknB (1,045 Å\(^2\)). Dimerization involving mutual exchange of structural elements has been seen previously in human ChPK2 (Supplementary Fig. S2)\(^\text{26}\). However, ChPK2 dimerization involves the transactivation loop (T-loop) of the C-lobe and not the N-lobe. Thus, the mode of dimerization of *Mtb*MaK is unique. Extensive inter-subunit interactions result in the formation of a tight dimer of *Mtb*MaK. This dimerization of *Mtb*MaK is signal independent and involves homotypic interactions.
2.49 mM for maltose (Fig. S7). This value is similar to the previously reported value for M. bovis BCG MaK (2.52 mM). To verify the role of the amino acids interacting with maltose in catalysis, we performed alanine-scanning mutagenesis and tested the ability of the mutants to phosphorylate maltose. Except for the P344A and K426A mutants, all other mutants could be expressed as soluble protein in E. coli. All nine mutants exhibited a dramatic loss in activity (activity less than 13% of the wild type) (Fig. 3f and Supplementary Table S4). Taken together, the structural observations coupled with results of mutagenesis studies suggest that Y222, D322, H324, S367, Y370, Y429, E430, Y433 and R438 are crucial for phosphorylation of maltose. These amino acids are conserved across MaK homologues from different sources (Supplementary Fig. S3).

The structure of the binary complex of MtbMaK with maltose helps explain the structural basis for a maltose-specific phosphorylation by MaK. The aromatic ring of Y433 stacks above the glycosyl moiety with the reducing end (Fig. 3e). Specifically, the C1 atom of the sugar is 3.5 Å away from the aromatic ring. In its β form, the O1 atom of maltose would either sterically clash with the aromatic ring of Y433 or result in non-productive binding. This is because stacking of Y433 with the glycosyl moiety brings catalytically critical residues in proximity of maltose (Fig. 3b). A β form of the maltose would not permit this stacking. In addition, the catalytic machinery is positioned for phosphorylation of the O1' atom of the α isomer. The O1' atom of maltose’s β form would be positioned far away from D322 to permit phosphorylation at this position.

Nucleotide-binding site of MtbMaK. Although there is no significant sequence conservation between MtbMaK and its homologues over the entire length of the protein, some conservation around the ATP-binding site is observed (Fig. 4a). To gain insights into the ATP-binding site of MtbMaK, the nucleotide-binding site of aminoglycoside phosphotransferase (APH; PDB code 1J7U) was superimposed over the structure of MtbMaK. This region of APH superimposed over an equivalent region of MtbMaK with an r.m.s.d. of 2.6 Å between the Ca atoms of 90 matching residues. Using such an approach, AMPPNP-Mg of APH could be docked into the nucleotide-binding site of MtbMaK (Fig. 4b) and a putative mode of ATP binding by MtbMaK could be inferred. The mode of ATP binding was verified by performing alanine-scanning mutagenesis of residues of MtbMaK interacting with the docked AMPPNP-Mg moiety (Supplemental text; Fig. 4c and Supplementary Table S4). The analysis revealed that amino acids like E142, Q143 and S144 from the “EQS” loop that is conserved across all MaK homologues (Supplementary Fig. S3) are important for binding ATP.

Discussion
Our structural studies on MtbMaK unveil several new, previously unknown features of maltose kinases. Many kinases dimerize upon receiving a signal and activate cellular signaling pathways. Such dimerization usually involves the C-lobe. In contrast, MtbMaK undergoes signal-independent homotypic dimerization mediated by the N-lobe. Dimerization probably offers a means for MtbMaK to form a scaffold that could readily hetero-oligomerize with the...
upstream enzyme, TreS, of the TreS-MaK-GlgE-GlgB pathway (Supplementary Fig. S1). The premises for such a scaffolding role for MtbMaK can be found in the hetero-octameric complex of TreS-MaK reported previously\(^7\). Further support for the scaffolding role of MaK is provided by the fact that the mak gene is usually located immediately downstream of the treS gene and in some micro-organisms the two genes are fused into a single gene\(^16\). Using shape complementarity, a model for interaction of MaK with TreS could be built with the N-terminus of MaK placed in proximity of the C-terminus of TreS, depicting the closely linked nature and order of the genes. In this model, a dimer of MaK fits on each face of a tetramer of TreS (PDB code 4LXF; Fig. 4d). Such an arrangement of

Figure 4 | Nucleotide binding site of MtbMaK. (a). Sequence alignment of nucleotide binding site (NBS) of MtbMaK and its homologues. SSE of MtbMaK are labeled on top of the aligned sequences. Identical residues are highlighted in red, and other conserved residues are highlighted in yellow. (b). Stereo view of AMPPNP-Mg (green sticks and spheres, PDB code 1J7U) superimposed on the NBS of MtbMaK. The C atoms of "EQS" loop, HGD motif and DFE motif are colored in cyan, magenta and light green, respectively. Strands β\(^8\) and β\(^9\) from the other subunit of MtbMaK are colored in blue. The side chains of residues potentially interacting with AMPPNP-Mg are shown as sticks. The O and N atoms are colored in red and blue. (c). Mutagenesis of residues from the NBS. A bar graph of relative activity (%) of mutants compared to the wild type enzyme is shown. Error bars represent s.d. (n = 3). (d). Model of hetero-octameric complex of TreS with MaK. Potential interaction of a tetramer of TreS (green color, PDB code 4LXF) with two dimers of MtbMaK (purple color) is shown. Active sites are marked with a red star; putative path of product marked in yellow.
TRE-S-MaK subunits aligns the active sites of the two proteins in a manner that facilitates the transfer of the product of TRE5, maltose, into the active site of MaK for phosphorylation (Fig. 4d).

Scaffolding roles for kinases have been demonstrated previously. For example, the integrin-linked kinase (ILK) functions exclusively as a scaffolding kinase. ILK binds CH2 domain of α-parvin and integrin tails during recruitment of focal adhesion proteins29. However, ILK cannot catalyze phosphate-transfers due to a defunct active site. In contrast, MtbMaK is active and readily phosphorylates α-maltose. Since maltose-1-phosphate is toxic to mycobacterium, it needs to be converted into other metabolites quickly. This could be accomplished by assembling a super complex of TRE-S-MaK-GlgE as proposed previously7. Using shape complementarity a dimer of GlgE could be possibly fit on the un-occupied face of the MaK dimer (Fig. 4d) giving rise to a super complex in the order GlgE dimer – MaK dimer – TRE tetramer – MaK dimer – GlgE dimer. Thus, MaK kinase could possibly serve as a scaffold for the assembly of a super complex. However, biochemical evidence for a TRE-S-MaK-GlgE complex has not come forth as yet. Results of our preliminary studies on ability of MtbMaK to form a complex with MtbGlgE using GST-pulldown assays and in vitro binding studies of tag-less protein using Biacore suggests that unliganded recombinant proteins do not form a complex. Perhaps, additional factors like ligands or TREs are required for the formation of the complex.

The dimer of MtbMaK observed in the crystal structure probably represents only one of the conformations assumed by the protein. MtbMaK eluted in two peaks during size exclusion chromatography (SEC) runs. Subsequent HPLC analysis revealed a similar pattern (Supplementary Fig. S5A). The retention time of the larger peak suggested presence of a dimer of MaK, while the shorter peak consisted of monomers of MaK. Protein from both the peaks was active when tested for kinase activity (Supplementary Fig. S5B). Analysis of symmetry mates revealed that MtbMaK could possibly form a tetramer (Supplementary Fig. S6). PISA analysis suggested a Complexation Significance Score (CSS) of 0.769 for the tetrameric assembly, indicating the possibility of MtbMaK assuming a tetrameric biological assembly. These results are consistent with previously reported ability of MtbMaK to exist in different oligomeric states in solution7.

The structures of MtbMaK reported here could be used as guides to develop inhibitors that could potentially aid treatment of TB. Analogues of α-maltose and maltose-1-phosphate could serve as scaffolds that could be re-designed to increase potency and other drug-like properties. Further, the use of MaK inhibitors could be extended to control other pathogens like Pseudomonas spp. that harbor the conserved mak gene. In this context, lack of mak gene in mammalian systems could prove to be beneficial for achieving greater specificity.

Methods

Molecular cloning. The gene encoding the full length MtbMaK (RV0127) was amplified by polymerase chain reaction (PCR) from Mycobacterium tuberculosis H37Rv genomic DNA. The product was sub-cloned into expression vector pGEX-6p-1 (GE Lifesciences) after digestion with BamHI and XhoI (TaKaRa). Site-directed mutagenesis was performed by PCR using native MtbMaK expression plasmid as a template. All mutants were constructed as per manufacturer’s instructions. Clones were verified by sequencing the entire gene. Recombinant plasmid containing either native mak gene or the mutants was then transformed into E. coli BL21 (DE3) for protein expression. Primers used in molecular cloning are listed in Supplementary Table S3.

Protein expression and purification. Cells containing recombinant plasmid were grown in Luria Bertani medium at 37 °C until OD600 reached 0.8. After cooling the culture, GST-tagged MtbMaK protein expression was induced at 16 °C by addition of 0.5 mM Isopropyl β-D-thiogalactoside (IPTG). After 20 h of induction, cells were harvested by centrifugation and re-suspended in 1 × PBS, pH 7.4. MtbMaK was recovered by first lysing the cells using sonication and clarifying the lysate by centrifugation at 30,000 g for 30 min to remove cell debris. The supernatant containing soluble recombinant protein was subjected to GST-affinity chromatography. Glutathione Sepharose 4B beads mixed with the supernatant were washed with buffer to remove non-specifically bound proteins. To remove theGST tag, PreScission protease (GE Lifesciences) was added and the mixture incubated overnight at 4 °C. Tagless MtbMaK protein was eluted from the column by gravity, concentrated and injected on a Superdex 200 10/300 GL gel filtration column equilibrated with a buffer containing 20 mM Tris-HCl pH 8.0, and 150 mM NaCl. Fractions containing the dimer were pooled and concentrated to 10 mg/ml for crystallization. Mutant proteins were expressed and purified in a similar way.

Crystallization and structure determination. Crystallization screening experiments were first carried out at 16 °C by vapor diffusion method in 96-well plates using a Mosquito liquid handling system (TTP Labtech). Each crystallization drop contained 200 nL of MtbMaK protein (10 mg/mL) mixed with 200 nL of reservoir solution. Commercially available sparse matrix screens were used for screening crystallization conditions. Crystals grew in a variety of conditions containing 1 M Sodium Citrate-Tris, pH 5.5, and 1% (v/v) PEG3350. Crystals were optimized by varying the concentration of (NH₄)₂SO₄ and pH. Optimized crystals were soaked in the reservoir solution supplemented with 20% (v/v) glycerol for 10 s and flash frozen in liquid nitrogen. X-ray diffraction data were collected at 100 K on beamline BL17U at SPring-8 (Shanghai, China) and processed using HKL2000 (HKL Research, Inc.)30, using the native derivatives were prepared by soaking the native crystals in the reservoir solution supplemented with 1 mM HgCl₂ for 2 h prior to cryoprotection. Anomalous peak data were collected on beamline BL41XU at Spring-8 (Hyogo, Japan) and processed to 3.2 Å resolution. The structure of MtbMaK was determined by the single-wavelength anomalous diffraction (SAD) method, using the peak derivative. All the 3 potential mercury atoms covalently linked to three cysteine residues were located using SHELXD31, and the initial phases were calculated using PHENIX32. The structural model was initially built according to the electron density map and then refined using the native data collected to 2.4 Å resolution. Manual adjustments to the model were made using COOT33 and refined using PHENIX. The MtbMaK-maltose complex was prepared by soaking MtbMaK crystals in the reservoir solution supplemented with 10 mM maltose (Sigma-Aldrich) for 1 h. The diffraction data for the complex were collected on beamline BLSA at KEK (Tsukuba, Japan) and processed to 2.9 Å resolution. The MtbMaK-maltose complex structure was solved by the molecular replacement method using the molecular replacement program (MR) in CCP434, using the native structure as a search template. The complex structure was refined using the native structure as a reference model in order to restrain the dihedral angles. The final structural models of native MtbMaK and MtbMaK-maltose complex have Rmerge/Refl values of 0.207/0.244 and 0.239/0.281, respectively. Data collection and refinement statistics are listed in Table 1. Structural figures were prepared using PyMol (DeLano Scientific).

Enzyme activity assay. Kinase activity was estimated by a linked spectrophotometric assay that uses pyruvate kinase and L-lactate dehydrogenase as auxiliary enzymes to monitor the conversion of ATP to ADP with an observable decrease in the absorption of NADH at 340 nm35. 100 µL of the reaction mixture contained 2 µg MtbMaK, 10 mM MgCl₂, 1.5 mM ATP, and 5 mM maltose suspended in a buffer made up of 20 mM Tris-HCl, pH 8.0, 150 mM NaCl. U of pyruvate kinase, U of L-lactate dehydrogenase, 0.3 mM NADH and 2.5 mM phosphoenolpyruvate (all from Sigma-Aldrich), were added and the mixture incubated at 37 °C for 30 min. The change in A630nm value was measured using a VarioScanFlash spectrophotometer (Thermo Scientific) over the course of the incubation period. The velocity of formation of the product ADP was calculated. The Km value of MtbMaK for maltose was determined from the Lineweaver-Burk plot constructed by measuring enzyme activity using different concentrations of the substrate.

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**Author contributions**
J.L. and X.G. performed experiments, interpreted results and wrote the manuscript. N.S. analyzed data and participated in drafting the article for important intellectual content. W.C., Y.D. and X.X. collected crystallographic data and analyzed it. Z.R. and X.L. initiated the study, co-designed experiments, interpreted data and wrote the manuscript. All authors have contributed to, seen and approved the manuscript.

**Additional information**
Accession codes: The co-ordinates and structure factor files for apo- and maltose-bound MaK have been deposited in the PDB under accession codes 4O7O and 4O7P, respectively.

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