Acetyl-CoA-mediated activation of *Mycobacterium tuberculosis* isocitrate lyase 2

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Isocitrate lyase is important for lipid utilisation by *Mycobacterium tuberculosis* but its ICL2 isoform is poorly understood. Here we report that binding of the lipid metabolites acetyl-CoA or propionyl-CoA to ICL2 induces a striking structural rearrangement, substantially increasing isocitrate lyase and methylisocitrate lyase activities. Thus, ICL2 plays a pivotal role regulating carbon flux between the tricarboxylic acid (TCA) cycle, glyoxylate shunt and methylcitrate cycle at high lipid concentrations, a mechanism essential for bacterial growth and virulence.
The ability of Mycobacterium tuberculosis (Mtb) to preferentially utilise lipids as its carbon source is a metabolic feature that enables chronic infection. Isocitrate lyase (ICL) isoforms 1 and 2 (Supplementary Fig. 1) are key enzymes in this process, through their roles in the glyoxylate and methylcitrate cycles (Supplementary Fig. 2). Both enzymes are essential for in vivo growth and virulence, but most studies have focused on the roles and structure of ICL1, leaving our understanding of ICL2 hampered by the lack of structural, functional and mechanistic insight.

Herein, using X-ray crystallography, we report the first structures of ICL2. Enzymatic assays and molecular dynamics calculations reveal that ICL2 is activated by the binding of acetyl-CoA or propionyl-CoA. Our results provide strong evidence that ICL2 may act as a gate-keeping enzyme, allosterically regulating the glyoxylate shunt and the methylcitrate cycle, an essential mechanism during chronic infection when Mtb uses lipids as the primary carbon source.

Results
Structure of ligand-free ICL2. We determined the crystal structure of ligand-free ICL2 from Mtb CDC1551 at 1.8 Å resolution (Fig. 1a). The 766-residue ICL2 monomer crystallised as a tetramer, with four subunits forming an elongated structure (length ~200 Å). Each subunit comprises two distinct domains, with the N-terminal (residues 1–590) and the C-terminal (residues 603–766) domains connected by a flexible linker (residues 591–602) (Supplementary Fig. 3). The N-terminal domain contains the αβ-barrel core common to all ICLs and is packed similarly to that in Mtb ICL1. It also possesses an active site loop containing the conserved catalytic motif (KKCGH), as in ICL1. However, the ICL2 N-terminal domain possesses an additional helical substructure that is not present in Mtb ICL1 (helices α10–α16; residues 278–427) (Supplementary Fig. 3). While such a structural insert is uncommon in bacterial ICLs, it is often present in fungal homologues.

The C-terminal domain of Mtb ICL2 is unique to this isoform, with no sequence homology to known proteins. However, structural searches revealed similarities to members of the Gcn5-related N-acetyltransferase (GNAT) superfamily, despite showing only 5–15% sequence identity (Supplementary Fig. 4). The C-terminal domains from two monomers associate at each end of the ICL2 structure (Fig. 1c), forming a barrel-like structure. The association appears weak, as confirmed by small-angle X-ray scattering (SAXS), which gives an experimental scattering profile that can only be explained on the assumption that multiple conformations are present in solution (Supplementary Fig. 5). We assume that crystal packing has selected one of several accessible conformational states.

ICL2 is activated by acetyl-CoA and propionyl-CoA. The structural resemblance of the Mtb ICL2 C-terminal domain to members of the GNAT superfamily prompted us to investigate the potential modulation of ICL2 activity by acetyl-CoA, which is the main product of fatty acid β-oxidation. The activity of Mtb ICL2 was measured by a nuclear magnetic resonance (NMR)-based assay that directly monitors reaction turnover (Supplementary Fig. 6a) and a fluorescence-based continuous assay that relies on the reaction between glyoxylate and phenylhydrazine (Supplementary Fig. 6b). Both assays gave comparable results; in agreement with previous studies, ICL2 showed poor isocitrate lyase activity using DL-isocitrate as a substrate (Fig. 2a, Supplementary Fig. 7 and Supplementary Table 1). However, in the presence of acetyl-CoA, ICL2 displayed a remarkable 50-fold increase in catalytic efficiency (kcat/KM = 37,200 ± 6000 M−1 s−1 at 27 °C) (Fig. 2a, Supplementary Fig. 7 and Supplementary Table 1). The KM value for the cofactor acetyl-CoA is 2.9 ± 0.5 μM (Supplementary Fig. 8), indicating it is a relatively strong binder to Mtb ICL2. The presence of the inactive L-isomer of isocitrate did not affect the catalytic activity of Mtb ICL2 or its allosteric activation by acetyl-CoA (Supplementary Fig. 10). This increase in catalytic activity upon the addition of acetyl-CoA appeared to be specific to Mtb ICL2, as addition of acetyl-CoA to Mtb ICL1 has no effect on the catalytic activity (Supplementary Fig. 11).

We then tested the methylisocitrate lyase activity of Mtb ICL2; no methylisocitrate lyase activity was detected with Mtb ICL2 alone (Fig. 2b, Supplementary Fig. 7 and Supplementary Table 1).
Acetyl-CoA triggers striking conformational changes in ICL2.

To understand these results, we determined the crystal structure of \textit{Mtb} ICL2 in the presence of acetyl-CoA at 2.36 Å resolution (Fig. 1b). One molecule of acetyl-CoA binds to each of the C-terminal domains in the tetramer, inducing striking conformational changes. Acetyl-CoA binding is mediated by extensive hydrogen bond interactions, which are exclusively formed with residues in the C-terminal domains (Supplementary Fig. 12). In the acetyl-CoA-bound structure, the C-terminal domain from one monomer moves an average of 77 Å towards the centre of ICL2 and rotates ~176° to form a new dimer with the C-terminal domain from the opposing monomer (Fig. 1c). The two C-terminal dimers formed when acetyl-CoA binds seem to be more stable as indicated by the buried interface area (1105 Å²) compared with the area in the ligand-free form (975 Å²). These distinctive conformational changes seen in the crystal structures are confirmed by the SAXS scattering profiles for ICL2 in the presence of acetyl-CoA, which are markedly different from that of the ligand-free ICL2 (Supplementary Fig. 5).

Molecular dynamics (MD) simulations for both the ligand-free and acetyl-CoA-bound ICL2 structures were conducted to investigate the mechanism of the observed allosteric activation of \textit{Mtb} ICL2 upon acetyl-CoA binding. Both structures show similar overall residue flexibilities, with local changes in regions associated with the reorganisation of the C-terminal domains (Supplementary Fig. 13a). Upon binding of acetyl-CoA, the loop consisting of residues 637–643 becomes more rigid due to the formation of the new dimer interface between the C-terminal domains. On the other hand, residues 382–388 become slightly more flexible, due to loss of interactions with the C-terminal domains resulting from the conformational change. Comparing the average structures of the C-terminal domains obtained from MD simulations shows two regions with clear local conformational changes, including residues 635–640 and residues 730–739 (Supplementary Fig. 13b). Both regions are involved in the formation of the new dimer interface between C-terminal domains upon acetyl-CoA binding (Supplementary Fig. 13c). Comparison of the C-terminal domain dimers by superimposition at one monomer shows a rotation between the monomers upon acetyl-CoA binding (Supplementary Fig. 13d). Such rotation requires a displacement of ~26 Å at the start of the C-terminal domain (residue 603). However, due to the constraints from the length of the linker region, such large movements would be prohibited in the ligand-free conformation, making the repositioning of the C-terminal domains necessary in order to form the new dimers upon acetyl-CoA binding.

Comparison of the average conformations of the N-terminal domain (residues 1–590) obtained from MD simulations reveals that the only region with clear conformational change upon acetyl-CoA binding is in the active site loop (Supplementary Fig. 13e). The changes in the conformations of the active site loop were closely examined and compared with the conformations observed in crystal structures of isocitrate-bound ICL1 from \textit{Brucella melitensis} (Bm ICL1, PDB 3P0X). Results suggest that in acetyl-CoA-bound ICL2 the conformations of the active site loop are more similar to that found in the crystal structure of substrate-bound Bm ICL1 (Fig. 3a). The root-mean-square deviation (RMSD) values between the active site loop in ICL1 (residues 183–187) and that in both ligand-free and acetyl-CoA-bound ICL2 (residues 213–217) were calculated using the trajectories obtained from MD simulations. The distribution of RMSD values in acetyl-CoA-bound ICL2 suggests a shift in the active site loop conformation towards a more catalytically-relevant conformation akin to that in substrate-bound Bm ICL1 (Fig. 3b). This is also confirmed by replica exchange molecular dynamic simulations (REMD), which was used to examine the conformations sampled by the active site loop. Our results from the REMD simulations also indicate that the active site loop largely samples different conformations in the ligand-free and acetyl-CoA-bound states (Supplementary Fig. 14). This...
conformational shift likely contributes to the underpinning mechanism of the observed activation of ICL2 by acetyl-CoA.

The correlated residue motions during MD simulations reveal two regions with high correlation, which interestingly corresponds to the ICL2-unique helical substructure in the N-terminal domain (α10–α16, residues 278–427), and the C-terminal acetyl-CoA binding domain (Supplementary Fig. 15a). Residues involved in correlated motions with either of the two regions were identified (Supplementary Table 2), which show opposite patterns between ligand-free and acetyl-CoA-bound ICL. In the ligand-free enzyme, N-terminal domain residues are mostly affected by the motion of helices α10–α16 (Supplementary Fig. 15b), whereas in acetyl-CoA-bound ICL, residues from the N-terminal domain mostly move in correlation with the C-terminal domain. Two residues from the active site loop (residues 214 and 215) showed correlation with the C-terminal domain in acetyl-CoA-bound ICL2, indicating the repositioning of the C-terminal domains has an influence on the movement of the active site loop (Supplementary Fig. 15c).

**Discussion**

The regulation of carbon flux between the tricarboxylic acid (TCA) cycle and glyoxylate shunt is critical for *Mtb*, especially given its ability to utilise multiple carbon sources simultaneously. Recent reports have attempted to dissect the potential mechanisms that control carbon flux between the two cycles. None of these studies, however, have addressed the potential regulatory role(s) of ICL2, whose function in *Mtb* lipid metabolism remains poorly understood. We have shown that activation of the isocitrate lyase activity of ICL2 occurs upon acetyl-CoA and propionyl-CoA binding, thus uncovering the unique role this isoform plays in the allosteric regulation of the glyoxylate shunt at high lipid concentrations. It appears that ICL2 acts as a gatekeeping enzyme to shift the carbon flux between the TCA cycle and the glyoxylate shunt, especially when lipids are utilised as the main carbon source. In addition to its role in the glyoxylate cycle, we have also unravelled the role of ICL2 in the methylcitrate cycle. Although ICL2 does not appear to have any methylisocitrinate lyase activity in the absence of allosteric activators, we have shown that acetyl-CoA and propionyl-CoA switch on the methylisocitrinate lyase activity of ICL2. The allosteric activation of ICL2 in response to increasing cellular propionyl-CoA may be a mechanism to alleviate the toxicity of the methylcitrate cycle intermediates during growth on odd-chain fatty acids or cholesterol. Overall, these observations provide a molecular-level understanding of ICL2 function in *Mtb* metabolism. The structural and mechanistic details reported herein provide the basis for the development of inhibitors against both ICL isoforms as potential antitubercular agents.

**Methods**

**Recombinant protein production.** A synthetic gene encoding ICL2 of *M. tuberculosis* CDC1551 was obtained from Integrated DNA Technologies (Supplementary Table 3), and then subcloned into pYUB28b for protein production (Supplementary Table 4). Recombinant ICL2 was expressed in *Escherichia coli* BL21 (DE3) LOBSTR cells transformed with the pGro7 plasmid (Takara Bio Inc.) expressing GroEL/GroES chaperones under the araBAD promoter. ICL2 was produced by leaky expression at 37 °C in Terrific Broth media containing 50 μg/mL each of hygromycin and chloramphenicol. L-Arabinose (final concentration 0.1%) was added to the culture media when the cell density reached an OD600 of between 0.4 and 0.6. This was followed by further incubation at 37 °C for 16 h. The His6-tagged ICL2 was purified by immobilised metal affinity chromatography (IMAC) and size exclusion chromatography (Supplementary Methods). Purified ICL2 aliquots were flash-frozen in 20 mM HEPES pH 7.5, 150 mM NaCl and 1 mM β-mercaptoethanol and kept at −80 °C until use.

**Structural search.** The C-terminal domain of ICL2 (residues 595–764) was compared against the structures in the Protein Data Bank (PDB) using the DALI Structural Search online server (http://ekhidna2.biocenter.helsinki.fi/dali/) and the 3D-BLAST Protein Structural Search online server (http://3d-blast.life.nctu.edu.tw/)

**Enzyme kinetics using NMR spectroscopy.** Enzyme kinetics were measured using an NMR-based method developed in our laboratory. In brief, all NMR experiments were conducted at a 1H frequency of 500 MHz using a Bruker Avance III HD spectrometer equipped with a BBFO probe. The instrument was operated using TopSpin 3.1 software. Experiments were conducted at 300 K. The pulse tip-angle calibration using the single-pulse mutation method (Bruker “pulsecal” routine) was undertaken for each sample. Water suppression was achieved using the excitation sculpting method. Unless otherwise stated, the number of transients was 16, and the relaxation delay was 2 s. Reactions were initiated by the addition of Mett ICL2. The lag time between the addition of enzyme and the end of the experiment was usually 4 min. All measurements were performed in triplicate. All experiments were conducted in 50 mM Tris-D11 (pH 7.5) in 90% H2O and 10% D2O (500 μL volume). Five millimolar MgCl2 is also added to the reaction mixture.
**Protein crystallography.** Ligand-free ICL2 crystals were obtained by sitting drop vapour diffusion, using a protein solution comprising 8 mg/ml ICL2, 1 mM succinate and 1 mM MgCl₂ with the Morpheus screen. Cubic-shaped crystals typically grew in ~15 days using a precipitant comprising 9% w/v PEG 4000, 18% v/v glycerol, an amino acid mix (0.02 M each of L-glutamate, DL-alanine, glycine, DL-lysine, DL-serine) and 0.1 M MES/imidazole pH 6.9. Diffraction data were collected using the MX1 and MX2 beamslines at the Australian Synchrotron. All datasets were indexed and processed using XDS, and scaled with AIMLESS. Additive screens around the most promising condition using the Morpheus screen, resulted in a crystal that formed in the presence of 0.001 M sodium tungstate dihydrate. A new dataset was collected from this crystal and used to solve the structure. The structure was solved by molecular replacement with Phaser using isoceatic isyze of Aspergillus nidulans (PDB 1DQJ) as a search model. This resulted in a protein model that contained only the N-terminal domain. The initial structure was improved to obtain the complete protein model containing both N- and C-terminal domains by visual inspection and model building with cycles of automatic (phenix.autobuild) and manual (COOT) model building. This structure was refined at 2.9 Å resolution using REFMAC5.

Additional screens around the most promising condition using the Morpheus additive screen, resulted in a crystal that formed in the presence of the 0.001 M sodium tungstate dihydrate. A new dataset was collected from this crystal and used to solve the structure, using the aforementioned ligand-free ICL2 as a search model. The final structure was refined to 1.8 Å resolution, with crystallographic Rfree of 16.83% and Rfree of 20.25%. Water molecules were identified by their spherical electron density and appropriate hydrogen bond geometry with the surrounding structure. Despite the presence of succinate in the crystallisation drops, no clear electron density could be observed for this molecule. Full refinement statistics are given in Supplementary Table 6.

Co-crystals of ICL2 and acetyl-CoA complex were grown from a solution containing 6 mg/ml ICL2, 1 mM acetyl-CoA and 1 mM MgCl₂ (crystal form 1). The best diffracting crystals were obtained in a solution comprising 8% w/v PEG 4000, 16% v/v glycerol, amino acid mix (0.02 M each of L-glutamate, DL-alanine, glycine, DL-lysine, DL-serine) and 0.1 M MIES/imidazole pH 6.5. The structure was solved by molecular replacement with MOLREP using the ligand-free ICL2 structure as a search model. An initial partial model was obtained using just the N-terminal domain of ligand-free ICL2, followed by using this partial solution as a fixed model to search for the C-terminal domain. Model building using phenix.autobuild and COOT resulted in a complete model that was refined at 2.67 Å resolution with crystallographic Rfree of 21.87% and Rfree of 24.10%. The structure showed unambiguous electron density for acetyl-CoA in all four subunits, with no clear electron density for succinate.

In an additional experiment, 3-nitropropionic acid (3-NP), an inhibitor of ICL, was also added to the co-crystallisation drops containing ICL2, acetyl-CoA and MgCl₂ (crystal form 2). A crystal was obtained from a solution containing 8% w/v PEG4K, 16% v/v glycerol, alcohol mix (0.02 M each of 1.6-hexanediol, 1-butanol, (RS)-1.2-propanediol, 2-propanol, 1,4-butanediol, 1,3-propanediol) and 0.1 M MIES/imidazole pH 6.9, which diffracted to 2.36 Å resolution, and was solved using the form I structure as a search model. This structure, with crystallographic Rfree of 22.8% and Rfree of 23.24%, showed unambiguous electron density for acetyl-CoA in all four subunits, albeit with no clear electron density for either succinate or 3-NP. Full refinement statistics are shown in Supplementary Table 6. Given the higher resolution of the form II structure, we have used this to discuss the ICL2-acetyl-CoA complex throughout the paper. The PDB_redo program was used in the final stages of refinement for all structures. All structural figures in the paper are produced using Pymol.

**Small-angle X-ray scattering (SAXS) analyses.** ICL2 protein aliquots were extensively dialysed against 20 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol (v/v), and 1 mM DTT and 0.1 mM TCEP (tris(2-carboxyethyl)phosphine). This buffer was used to make the ligand solutions and to dilute protein samples. The 96-well plates containing the samples were mounted on a temperature-controlled mount at 283 K for autosampling and capillary flow data acquisition, comprising consecutive 1-ns X-ray exposures. SAXS data were collected on the Australian Synchrotron S8 SAXS beamline using the Phenix software suite,37,38 and MultiFoX servers39 were used to compute SAXS profiles of ICL2 structures (X-ray structure as well as calculated population-weighted ensembles) to the experimental profiles (Supplementary Table 7).

**Molecular dynamics simulations.** MD simulations were conducted using NAMD 2.1240 and trajectories were visualized and analysed in VMD.41 Crystal structures for ligand-free and acetyl-CoA-bound ICL2 obtained in this study were used as starting points for MD simulations. Initial force field topology and parameters for acetyl-CoA were obtained from CgenFF server (https://cgenff.paramchem.org)42-44, from which parameters were assigned by analogy to existing parameters in the force field. A penalty score is given to each assigned parameter and those with large penalty values were further refined by Force Field Toolkit. Additional explicit TIP3P water molecules were added to solvate the protein molecules in a water box in VMD. Na⁺ and Cl⁻ ions were added to balance the net charge of the water box. MD simulations were conducted with CHARMM36 force field45 at a constant temperature and pressure (298 K and 1 atm). The cutoff distance for the van der Waals interactions was set to 12 Å. In each simulation, the system was first minimised for 5000 steps followed by dynamics simulation conducted with 2 fs time steps. Three MD simulations were conducted for each of ligand-free and acetyl-CoA-bound ICL2, initiated with different random seeds. MD simulations were conducted for 274.5, 277 and 279.9 ns for ligand-free enzyme, and 232.4, 236.6 and 236 ns for acetyl-CoA-bound ICL2. The total simulation time for ligand-free enzyme is 828.4 ns and for the acetyl-CoA-bound enzyme is 705 ns. Considering there are four identical chains on the symmetrical homotramer of Mtb ICL2 in each simulation, the total amount of simulation time for each protein chain is 3.3 and 2.8 ps for ligand-free and acetyl-CoA-bound protein, respectively. The residue fluctuations during MD simulations were analysed. The fluctuations were obtained by calculating the RMSD values for each residue, using the side chain atoms, between each frame of trajectory. The correlation coefficients between the residue fluctuations were then computed as defined by the Pearson product-moment correlation coefficient, in equation

\[ R_{xy} = \frac{C_{xy}}{\sqrt{C_{xx} \times C_{yy}}} \]

where \( C_{xy} \) is the covariance of \( x \) and \( y \), \( C_{xx} \) and \( C_{yy} \) are the variance of \( x \) and \( y \), respectively. The calculations and analyses of the correlation matrices were conducted using python v3.6.5 and Jupyter Notebook v5.0.

**Replica exchange molecular dynamics (REMD).** REMD simulations (parallel tempering) were conducted using NAMD 2.1240 and trajectories were visualized and analysed in VMD.41 The same starting points for ligand-free and acetyl-CoA-bound ICL2 as in the MD simulations were used. Two sets of REMD were conducted for each of the ligand-free and acetyl-CoA-bound ICL2 states. The first set is set up with 10 replicas exchanging between a lower-temperature range of 300–310 K, and the second set contains 10 replicas exchanging at a higher-temperature range of 310–320 K. Attempts to exchange were made every 1000 steps of simulation. For the lower temperature REMD set, 51.2 ns of trajectories per replica were collected for the ligand-free system and 48.8 ns per replica for acetyl-CoA-bound system. For the high temperature REMD, 23.3 ns per replica were collected for the ligand-free system and 22.7 ns per replica were collected for the acetyl-CoA-bound system.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 6EDW, 6EDZ and 6EE1. The source data underlying Fig. 2a and b and Supplementary Figs. 6a, 7a, 7b. 8a, 8b, c, 9a, 9b, c, d, 10 and 11 are provided as a Source Data file. Other data are available from the corresponding authors upon reasonable request.

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Author contributions

R.P.B. and G.B. cloned the constructs and purified ICL2. R.P.B. and G.B. performed protein crystallography. R.P.B. and G.B. solved the ICL2 structures with the help of J.R. G.B. performed SAXS studies. R.P.B. performed enzymatic assays by NMR with the help of B.B.C.K. B.B.C.K. conducted enzymatic assays by UV/VIS spectroscopy with the help of A.J.C. W.F. performed molecular dynamics calculations. R.P.B., W.J., B.B.C.K., J.R., J.S., G.B. and I.K.H.L. analysed the data. J.S., G.B. and I.K.H.L. designed and supervised the studies. G.B. and I.K.H.L. wrote the paper with input from A.J.C.W.

Competing interests

The authors declare no competing interests.
Additional information

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