Geranyl-CoA carboxylase (GCC) is essential for the growth of Pseudomonas organisms with geranic acid as the sole carbon source. GCC has the same domain organization and shares strong sequence conservation with the related biotin-dependent carboxylases 3-methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC). Here we report the crystal structure of the 750-kDa $\alpha_6\beta_6$ holoenzyme of GCC, which is similar to MCC but strikingly different from PCC. The structures provide evidence in support of two distinct lineages of biotin-dependent acyl-CoA carboxylases, one carboxylating the $\alpha$ carbon of a saturated organic acid and the other carboxylating the $\gamma$ carbon of an $\alpha$-$\beta$ unsaturated acid. Structural differences in the active site region of GCC and MCC explain their distinct substrate preferences. Especially, a glycine residue in GCC is replaced by phenylalanine in MCC, which blocks access by the larger geranyl-CoA substrate. Mutation of this residue in the two enzymes can change their substrate preferences.
Terpenes are a common biosynthetic precursor of many biological compounds, including hormones and cholesterol, and have long been studied for their many medicinal properties. Monoterpenes are the simplest terpenes and are formed by the joining of two five-carbon isoprene units. Terpenes are modified and cyclized in various ways to form numerous terpenoids, including hormone precursors, scents, and flavourings. Isopropanylation is also used for localizing proteins to the cell membrane. In certain bacteria, terpenes can be used as a primary carbon source and metabolized to enter the tricarboxylic acid cycle as acetyl-CoA.

Geranyl-CoA carboxylase (GCC) activity was first purified from a Pseudomonas organism grown with either the related acyclic monoterpenoid citronellol or geranic acid as the sole carbon source. GCC also possesses 3-methylcrotonyl-CoA carboxylase (MCC) activity, and 3-methylcrotonyl-CoA is an intermediate in the complete catabolism of citronellol and geranic acid. In comparison, the authentic MCC enzyme cannot carboxylate the larger geranyl-CoA substrate. GCC and other enzymes involved in the first half of the geranic acid degradation pathway are encoded by the acyclic terpene utilization operon, while MCC and those involved in the second half are encoded by the leucine/isovalerate utilization operon. GCC is found in Pseudomonas and a collection of other bacteria. MCC is conserved from bacteria to humans, and is crucial for leucine metabolism. Deficiencies in MCC activity in humans are linked to methylcrotonylglycinuria and other serious metabolic diseases.

GCC and MCC are members of the biotin-dependent carboxylase superfamily. Both consist of two subunits, \( \alpha \) and \( \beta \), and their holoenzymes are 750-kDa hexamers. The \( \alpha \) subunit contains the biotin carboxylase (BC), BC–CT interaction (BT), and the biotin carboxyl carrier protein (BCCP) domains. The \( \beta \) subunit contains the N and C domains of carboxyltransferase (CT). The \( \alpha \) subunits of Pseudomonas GCC and MCC share 51% amino acid sequence identity, and their \( \beta \) subunits share 46% identity. These two enzymes therefore have high sequence conservation, but the molecular basis for their distinct substrate preferences is currently not known. In fact, many of the GCC/MCC enzymes in the sequence database have been misannotated (see below).

Propionyl-CoA carboxylase (PCC) shares the same domain organization as GCC and MCC. However, our recent crystal structures of MCC and PCC showed that their holoenzymes have strikingly different overall architectures. Moreover, the connectivity between the N and C domains is different in the two \( \beta \) subunits. These results led to the hypothesis that there are two distinct lineages of biotin-dependent acyl-CoA carboxylases, those that carboxylate the \( \alpha \) carbon of the organic acid (PCC, acetyl-CoA carboxylase) and those that carboxylate the \( \gamma \) carbon of an \( \alpha \)-\( \beta \)-unsaturated acid (GCC, MCC) (Fig. 1a). A prediction of this hypothesis is that GCC should have a similar \( \beta \) subunit connectivity as MCC, and that the GCC holoenzyme may have a similar architecture as MCC. Here we report the crystal structure of the GCC holoenzyme, as well as mutagenesis studies to define the molecular basis for the distinct substrate preferences of GCC and MCC.

**Results**

**Structure of GCC \( \beta \) subunit hexamer.** We first determined the structure of Pseudomonas aeruginosa GCC (PaGCC) \( \beta \) subunit at 2.4 Å resolution. The current atomic model has good agreement with the crystallographic data and the expected geometric parameters (Table 1). The structure confirms our hypothesis, showing that GCC\( \beta \) has the same connectivity between its N and C domains as that of MCC\( \beta \) (Fig. 2a). The overall structures of the two subunits are similar, with root mean squared distance of 1.2 Å for 464 equivalent Cz atoms between them, consistent with their strong sequence conservation. The overall structures of the two \( \beta \) hexamers are similar to each other as well, with root mean squared distance of 1.3 Å for 2,866 equivalent Cz atoms (Fig. 2b).

![Figure 1](image-url)  
**Figure 1** | The metabolic pathways involving GCC and MCC. (a) The metabolism of acyclic terpenes requires the activity of both GCC and MCC, while the metabolism of the amino acid leucine requires only MCC. The Atu and Liu pathways are sequentially similar, involving dehydrogenase, carboxylase, hydratase and lyase activities. (b) Domain organization of GCC, MCC and PCC \( \alpha \) and \( \beta \) subunits. The various domains are labeled. Atu, acyclic terpene utilization; Liu, leucine/isovalerate utilization.
However, there are regions of substantial conformational differences between the two subunits. While the connectivity in PaGCCβ is the same as in MCCβ, the linker between the N and C domains has a different conformation (Fig. 2a). In addition, the C-terminal segment of PaGCCβ (residues 526–538) contacts the N domain, while that of MCCβ contacts the C domain. The long helix at the N-terminal end of PaGCCβ is disordered in one of the three β subunits in the asymmetric unit (Fig. 2b), although this helix is fully ordered in the GCC holoenzyme structure. Finally, structural differences in the active site region of the two enzymes define their distinct substrate preferences (see below).

**Structure of 750-kDa GCC holoenzyme.** We next determined the crystal structure at 3.1 Å resolution of the *Pseudomonas aeruginosa* geranyl-CoA carboxylase; PaGCC, *Pseudomonas fluorescens* geranyl-CoA carboxylase; r.m.s., root mean squared. *The numbers in parentheses are for the highest resolution shell.*

| Table 1 | Summary of crystallographic information. |
|-----------------|-----------------|-----------------|
| **Data collection** | PaGCCβ hexamer | PaGCC holoenzyme |
| Space group | P6₁,22 | P4₁,2,2 |
| Cell dimensions | 105.7, 105.7, 550.8 | 234.6, 234.6, 274.4 |
| a, b, c (Å) | 90, 90, 120 | 90, 90, 90 |
| Resolution (Å)* | 30–2.4 (2.53–2.4) | 50–31 (3.26–3.1) |
| Rmerge | 8.4 (43.2) | 12.2 (>100) |
| CC1/2 | 0.48 |
| Completeness (%) | 100 (99) | 98 (95) |
| Redundancy | 4.5 (4.5) | 3.7 (3.6) |

**Refinement**

| Parameter | Value |
|-----------------|-----------------|
| Resolution (Å) | 47–2.4 | 50–3.1 |
| No. of reflections | 68,848 | 128,390 |
| Rwork/Rfree | 21.6/26.2 | 19.6/22.8 |
| No. of atoms | Protein 11,411 | 25,281 |
| | Ligand/ion 0 | 0 |
| | Water 172 | 0 |
| B-factors | Protein 66 | 99 |
| | Ligand/ion – | – |
| | Water 52 | – |
| R.m.s. deviations | Bond lengths (Å) 0.013 | 0.011 |
| | Bond angles (°) 1.6 | 1.5 |

PaGCC, *Pseudomonas aeruginosa* geranyl-CoA carboxylase; PfGCC, *Pseudomonas fluorescens* geranyl-CoA carboxylase; r.m.s., root mean squared. *The numbers in parentheses are for the highest resolution shell.*

The distance between the BC and CT active sites is ~80 Å in the GCC holoenzyme (Fig. 3a). Therefore, the entire BCCP domain must translocate during GCC catalysis, as is the case with MCC (ref. 19), PCC (ref. 18), long-chain acyl-CoA carboxylase20, pyruvate carboxylase21–24, and urea carboxylase25. The BCCP-biotin is bound in the active site of the CT domain in the current structure, at roughly the same position as that in the MCC holoenzyme (Fig. 3a). In four of the six BCCP domains in the GCC holoenzyme, a portion of the structure located farthest from the holoenzyme core is disordered. In comparison, only one of the six BCCP domains is ordered in the MCC holoenzyme (Fig. 3a)19.

We also carried out cryo electron microscopy (cryoEM) studies on the PIGCC holoenzyme and produced a reconstruction at 5.6 Å resolution (Fig. 4a,b). The crystal structure and the cryoEM reconstruction are in excellent agreement with each other overall. The cryoEM studies therefore provide important, independent confirmation that GCC assumes the same structure in solution. The four B subdomains of BC that are disordered in the crystal are observed by cryoEM. However, the two B subdomains observed in the crystal show a different conformation in the cryoEM reconstruction. The disordered segments of the BCCP domains in the crystal are also observed by cryoEM (Fig. 4a), but the overall positions of the BCCP domains in the CT active site are essentially the same in the crystal structure and cryoEM reconstruction.

**A BT domain in the structure of GCC.** The sequences of the BT domains are not well conserved among GCC, MCC and PCC, in contrast to the BC and CT domains (Supplementary Figs 1,2). The structure of the BT domain in GCC consists of a long α-helix (αV) surrounded by an eight-stranded anti-parallel β-barrel (β22–β29, Fig. 5a). In comparison, the helix of the MCC BT domain is shorter (Fig. 5b), and its β-barrel is missing a strand (β24, Fig. 5a), although this might be unique to *P. aeruginosa* and closely related MCCs (Supplementary Fig. 1)19. In this regard, the structure of the GCC BT domain appears more similar to that of the PCC BT domain (Fig. 5c). On the other hand, the long loop connecting the helix to the first β strand, the ‘hook’18, has different conformations in the three BT domains, and mediates unique interactions that may help to determine the relative positions of the α and β subunits in these holoenzymes. These unique interactions also ensure the fidelity of each holoenzyme, such that a GCC-MCC hybrid cannot be produced.

The C-terminal segment of this hook in the GCC BT domain contains a short β-strand and establishes similar interactions with the β subunit as observed in MCC (Fig. 5a). The first residue of this β-strand is Trp483, mostly conserved among GCC and MCC enzymes (Trp543 in PaMCC, residue numbers according to human MCC, Supplementary Fig. 1). The side chain is buried at the interface between the N and C domains of the β subunit. This part of the hook is also located near the long helix (α0) at the N-terminal end of the β unit, a structural feature that is absent in PCC.

The N-terminal segment of the hook in the MCC BT domain is much longer, and establishes interactions with the linker between the N and C domains of the β subunit (Fig. 5b). In fact, the conformational differences for this linker between GCC and MCC (Fig. 2a) are likely due to this interaction with the BT domain in MCC, as the conformation of the linker in the GCC holoenzyme would clash with this part of the hook in the MCC holoenzyme (Fig. 5b). This different conformation of the linker, by up to 10 Å, is in turn coupled to the different positioning of the C-terminal segments of the β subunits. The C-terminal segments of two monomers in a β₆ dimer are swapped with each other.
between GCC and MCC (Fig. 5b), again illustrating the dramatic structural plasticity among these highly conserved enzymes.

Molecular basis for substrate preference. Based on the binding modes of CoA to MCC (ref. 19) and crotonyl-CoA to glutaconyl-CoA decarboxylase α (ref. 26), we built a model for the binding mode of geranyl-CoA in GCC (Fig. 6a). There is a large, mostly hydrophobic pocket at the bottom of the active site in GCC that can accommodate the larger geranyl group. The pocket is formed primarily by residues in helices α3, α4 and α5 in the N domain, with only minor contributions from residues in the C domain. Interestingly, the side chain of Phe191 in MCCβ is located in this pocket and clashes with the second isopentenyl group of geranyl-CoA. The equivalent residue in GCC is Gly162. There are also differences in the main-chain atoms of the residues in this region, and MCCβ contains a three-residue insertion here (Supplementary Fig. 2). Overall, these structural differences reduce the size of the active site pocket in MCCβ, thereby defining the molecular basis for why MCC is not active toward the larger geranyl-CoA substrate. Conversely, the larger pocket in GCC can accommodate both geranyl-CoA and 3-methylcrotonyl-CoA, and therefore it can be active toward both substrates.

To test these structural observations, we created the G162F mutant of GCCβ and the F191G mutant of MCCβ. The G162F mutation destabilized the GCC holoenzyme and it dissociated during gel filtration unless stabilized by higher salt concentrations. The activity of wild-type GCC toward the geranyl-CoA substrate (Fig. 6b) is about fourfold lower than that of wild-type MCC toward the 3-methylcrotonyl-CoA substrate (Fig. 6c). Wild-type GCC showed very weak activity towards 3-methylcrotonyl-CoA. The $K_m$ of this reaction is very high, suggesting that binding of the smaller CoA compound is not optimal in the GCC active site.

As predicted by the structure, the G162F mutant of GCC is catalytically inactive toward geranyl-CoA, while the F191G mutant of MCC showed appreciable activity towards this substrate, about fivefold lower than wild-type GCC (Fig. 6b). Conversely, the G162F mutant of GCC demonstrates stronger activity towards 3-methylcrotonyl-CoA than wild-type GCC, while the F191G mutant of MCC is essentially inactive (Fig. 6c).

Discussion

The kinetic parameters reported here for the wild-type enzymes are generally similar to those from previous studies on GCC and MCC. An apparent $K_m$ value of 50 μM was determined from our studies on PaGCC (Fig. 6b). However, only ~25% of the geranyl-CoA compound we used was in the cis configuration. Therefore, the actual $K_m$ value is likely ~12 μM, comparable to the 9 μM reported earlier for PaGCC (ref. 8). The $K_m$ value for PaMCC was 69 μM based on our studies (Fig. 6c), while the earlier study reported 10 μM as well as cooperative behaviour (Hill coefficient 2.3). However, in a more recent paper27, the $K_m$ was found to be 220 μM and no cooperativity was reported. These other kinetic studies are based on the CO2 fixation assay, while our studies are based on the coupled enzyme assay monitoring ATP hydrolysis. Some of the differences in the kinetic parameters could also be due to the differences in the assay protocols.

Since GCC, MCC, and PCC have similar domain organizations (Fig. 1b) and conserved sequences, we have found that they are frequently misannotated in the sequence database. The structural information on the three holoenzymes suggests guidelines for how they can be classified based on their sequences. PCCβ lacks an extended α-helix (~30 residues) at the N terminus and can be separated from GCCβ and MCCβ based on this. The mitochondrial targeting sequence of animal PCCβ should be excluded from this consideration. MCCβ has a Phe residue in the active site region of the N domain, and this residue is replaced a Gly in GCCβ. MCCβ also has a short insertion of 3 residues just before this Phe residue compared with GCCβ. With these guidelines, it should be possible to accurately annotate the different enzymes.

Overall, our studies on GCC, MCC and PCC support the presence of two distinct ligands of biotin-dependent acyl-CoA carboxylases. The structural differences among these enzymes also have wide implications for the relationship between sequence conservation and structural similarity. The differences in substrate preferences between GCC and MCC are defined by differences in their sequences, and consequently structures, in the active site region.

Methods

Protein expression and purification. GCC holoenzymes were overexpressed using a bi-cistronic plasmid, with GCCα (untagged) placed downstream of GCCβ (N-terminal His-tagged) in a similar fashion to the MCC and PCC holoenzymes18,19. The hexa-histidine tag was not removed for crystallization. PaGCC holoenzyme was overexpressed in Escherichia coli BL21 Star (DE3) cells (Novagen) in the presence of 1 mM IPTG (Gold Biotechnology, Inc) to induce expression and incubated at 20°C overnight. The soluble protein was eluted from Ni-NTA beads (Qiagen) and further purified by gel filtration in a column buffer.
Figure 3 | The crystal structure of the GCC holoenzyme. (a). Overlay of the structure of PfGCC holoenzyme (in colour) with that of PaMCC holoenzyme (PDB entry 3U9T, in grey)\textsuperscript{19}, viewed down the two-fold symmetry axis of a β subunit dimer (black oval). Domains in the α and β subunits in the top half of the structure are coloured as in Fig. 1b for GCC. The α and β subunits in the bottom half are coloured in magenta and green, respectively. (b). Overlay of the structure of three BC domains in PfGCC (in red) with that of the BC domains in PaMCC (in grey), viewed down the three-fold symmetry axis of the holoenzyme (black triangle). The differences in the conformations of the BC domains are indicated with the red arrows, corresponding to a rotation of \(9^\circ\). Only one of the B domains of BC in the \(α_3β_3\) asymmetric unit is ordered in PfGCC.

Figure 4 | The cryo electron microscopy structure of the GCC holoenzyme. (a). CryoEM reconstruction of PfGCC at 5.6 Å resolution, shown as a transparent surface (in grey) and viewed in the same orientation as Fig. 3a. The crystal structure of PfGCC can be readily fit into the cryo EM density. Several of the B domains of BC and the disordered segments of BCCP in the crystal structure are observed in the cryo EM density, indicated with the red arrows. The conformation of the two ordered B domains in the crystal is somewhat different from that observed by EM. (b). Cryo EM reconstruction of PfGCC, viewed down the three-fold symmetry axis of the holoenzyme (black triangle). Produced with Chimera\textsuperscript{38}. 
converting 25 mM Tris (pH 7.4), 250 mM NaCl and 2 mM DTT. The purified protein was concentrated to 12 mg ml\(^{-1}\), supplemented with 5% (v/v) glycerol, flash-frozen with liquid nitrogen and stored at \(\sim 80\) °C. An SDS gel of this sample showed that the \(\beta\) subunit was present in significantly substoichiometric amounts.

\(P.\,\text{fluorescens}\) GCC (PaGCC) holoenzyme was expressed in the same condition as the PaGCC holoenzyme and purified by gel filtration in a column buffer containing 20 mM Tris (pH 8.0) and 60% (v/v) MPD. Crystals were grown by using the microbatch-under-precipitant method with the hanging-drop vapour diffusion method.

**Cryoelectron microscopy.** Before data collection, the microscope was carefully aligned, and beam tilt was postcorrected with the UCSF software\(^{34}\). The electron gun was set to \(\sim 8\) counts per pixel per s on the camera which corresponds to \(\sim 10 \text{e}^{-} \text{Å}^{-2} \text{s}^{-1}\) on specimen when including the electrons uncounted by the K2 camera. Image stacks were recorded at 4 frames per sec for 10 s. After drift correction with the UCSF software\(^{34}\), the first 12 frames of each image stack (movie) were merged to generate a final image with a total dose of \(\sim 30 \text{e}^{-} \text{Å}^{-2}\) of the sample.

**CryoEM image processing.** A total of 41,271 particle images were picked from 1,149 images automatically with the *Dog Picker* program\(^{35}\). The underfocus values of these images were determined to be between 1.06 to 3.4 μm using CTFFIND3\(^{36}\). Image processing and reconstruction were carried out using RELION\(^{37}\). Two-dimensional classification was first used to screen particles. After 25 iterations, 16 two-dimensional classes with 31,206 particles were picked from the total 30 classes. Three-dimensional (3D) classification was then performed with RELION to classify all particles into three groups. However, the reconstructions from these three groups are nearly identical. We therefore used all 31,206 particles for 3D refinement, which started from a featureless starting model generated by...
Figure 6 | Molecular basis for the different substrate preferences of GCC and MCC. (a). A model for the binding mode of the geranyl-CoA substrate (in black) in the active site of GCC (cyan) for the N domain and yellow for the C domain of the β subunits. The structure of MCC is superimposed and shown in grey. The side chain of Phe191 of MCCβ is shown as a stick model (grey), and the Ca atom of Gly162 of GCCβ is shown as a sphere (cyan). (b). Catalytic activities of wild-type GCC and MCC, the G162F mutant of GCC and the F191G mutant of MCC toward the 3-methylcrotonyl-CoA substrate. The error bars represent the standard deviations from three separate experiments. The kinetic parameters are shown in the inset. (c). Catalytic activities of wild-type GCC and MCC, the G162F mutant of GCC and the F191G mutant of MCC toward the 3-methylcrotonyl-CoA substrate.
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**Author contributions**

A.R.J. and C.S.H. expressed, purified and crystallized the proteins. A.R.J., C.S.H. and L.T. determined the structures. A.R.J. carried out the mutagenesis and kinetic assays. Z.H.Z. carried out the cryoEM studies. Z.H.Z. and L.T. supervised the project, analyzed the data, and wrote the manuscript. All other authors commented on the manuscript.

**Additional information**

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