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Structure of *Aspergillus fumigatus* Cytosolic Thiolase: Trapped Tetrahedral Reaction Intermediates and Activation by Monovalent Cations

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ABSTRACT

Cytosolic thiolase (CT) catalyzes the reversible Claisen condensation of two molecules of acetyl-CoA to produce acetoacetyl-CoA. The reaction cycle proceeds via a ping-pong mechanism involving an acetylated enzyme intermediate and two separate oxyanion holes which stabilize negatively charged reaction intermediates. This is the initial step in the synthesis of ergosterol in the prominent fungal pathogen, *Aspergillus fumigatus*, and is essential for the growth and survival of the organism. Here, we present crystal structures of *A. fumigatus* CT in liganded and apo forms, and in complex with different monovalent cations. Careful observation of the electron density at the active sites of two different afCT structures crystallized in the presence of acetyl-CoA shows that our crystals have trapped various stages of thiolase catalytic cycle, including two tetrahedral reaction intermediates that have previously eluded structural characterization. Unexpectedly, we have also shown that afCT is activated by monovalent cations, a biochemical property previously thought to apply only to the mitochondrial biosynthetic thiolase, with a preference for potassium ions. Structures of fungal CT provide valuable insight into the thiolase reaction cycle and allosteric activation of members of this class of enzymes by monovalent cations.

KEY WORDS: *biosynthetic thiolase, acetoacetyl-CoA, Claisen condensation, tetrahedral reaction intermediate, monovalent cation activation*

INTRODUCTION

Ergosterol is the main fungal cell-membrane sterol – essential for fungal cell viability and survival, and is lacking in host cell membranes, making it an attractive antifungal target. Antifungals targeting ergosterol are central to the treatment of invasive aspergillosis (IA); a debilitating condition caused by *Aspergillus spp.* associated with high morbidity and mortality rates, and an increasing incidence in recent decades [1-5]. First-line anti-*Aspergillus* drugs target ergosterol, either directly in the membrane, or by inhibiting its synthesis. Amphotericin B binds ergosterol directly, disrupting membrane function [6-8]. Although it has been used to treat IA for over four decades, it has largely been superseded by the azole class of antifungals due to its poor side-effect profile [9]. Triazole antifungals

target 14-α-lanosterol demethylase (*ERG11*), inhibiting ergosterol synthesis at the conversion of lanosterol to C14-demethyl-lanosterol. Voriconazole is currently recommended for first-line treatment of IA in most cases [10]. Unfortunately, triazole antifungals are also associated with significant side effects including liver toxicity, psychosis, and visual disturbances [9, 11]. More importantly, the past two decades have seen an alarming increase in the incidence of azole-resistant isolates of *A. fumigatus* – the main causative agent of IA – worldwide. Azole-resistant strains of *A. fumigatus* have emerged both in the clinic and in the environment and are associated with poorer treatment outcomes [12-14].

Because of its essential role in fungal growth and relevance to the action of currently available antifungals, the biosynthesis of ergosterol is an essential area of study to improve current antifungals or uncover new targets in the synthetic pathway. In *A. fumigatus*, squalene is converted to ergosterol via approximately 12 distinct enzymes [15]. Squalene is produced from mevalonate – the precursor for all isoprenoids, which include many important organic molecules such as sterols (including ergosterol), quinones, polyprenyl alcohols, heme A and the post-translational prosthetic groups farnesyl and geranylgeranyl [16-17]. Mevalonate is produced in the cytosol from acetyl-CoA (Ac-CoA) via three catalytically independent steps (Figure 1). The first step is catalyzed by an acetoacetyl-CoA (AcAc-CoA) thiolase (ACAT). Therefore, synthesis of ergosterol, and all other sterols, starts with cytosolic ACAT.

Thiolases are ubiquitous enzymes involved in the transfer of acyl groups between acyl-CoA derivatives. There are two functional classes: type I thiolases, referred to as 3-ketoacyl-CoA thiolases (KAT), and type II thiolases which are referred to as acetoacetyl-CoA thiolases (ACAT) or acetyl-CoA acetyl-transferases [18-19]. ACATs studied thus far are typically homotetramers, while KATs are homotetramers or homodimers. KATs can accept acyl-CoA derivatives with a variety of chain lengths and are localized to mitochondria or peroxisomes where they are involved in β-oxidation of fatty acids [18]. ACATs are cytosolic, mitochondrial or peroxisomal [20] and are specific for short chain acyl-CoA substrates (C2-C4); catalyzing the formation or degradation of AcAc-CoA from Ac-CoA. Although the degradative reaction is entropically favored, high cellular concentrations of Ac-CoA derived from cellular catabolism along with rapid turnover of AcAc-CoA by the next enzyme in the pathway are postulated to drive the reaction in the biosynthetic direction [21-25]. ACATs are therefore often referred to as biosynthetic thiolases. While cytosolic ACAT is essential for sterol synthesis, mitochondrial ACAT is essential for ketone body metabolism [26-28]. In addition, mammalian mitochondrial ACAT (referred to in the literature as T2) differs from its cytosolic counterpart (referred to simply as cytosolic thiolase; CT) regarding its substrate specificity and dependence on ions for activity. Unlike CT, T2 has been shown to turn over 2-methylacetoactyl-CoA, implicating it in isoleucine catabolism [27-28]. Also, T2 has K^+ and Cl^- ion binding sites near its active site and its catalytic activity is increased several fold in the presence of K^+ ions, whereas the activities of bacterial and mammalian CTs studied thus far are ion independent [29-30]. K^+ dependent stimulation of thiolase activity is used as a tool for diagnosis of human T2 deficiency, a well characterized disorder presenting with episodes of acute ketoacidosis, caused by mutations in the gene encoding for T2 [27, 31-32].

The cytosolic ACAT catalytic cycle has been studied in detail in the bacterium *Zoogloea ramigera* [22, 24, 33-37], and involves the sequential binding of two Ac-CoA molecules to a single catalytic pocket (Figure 2). Upon binding of the first Ac-CoA, the acetyl group is transferred from the CoA to the catalytic cysteine, Cys89, forming an acetylated protein intermediate (Figure 2, stages 1-4). His348 is postulated to deprotonate Cys89, activating it for nucleophilic attack on the carbonyl of the acetyl moiety. Another Ac-CoA then binds and a Claisen condensation reaction occurs, involving transfer of the acetyl group from the enzyme, forming AcAc-CoA (Figure 2, stages 5-8). Cys378 is the active site base, abstracting a proton from C2 of the second acetyl moiety, activating it for nucleophilic attack on the carbonyl of the first acetyl moiety [34, 37]. In both acetyl-transfer reactions, the reaction must proceed via a tetrahedral intermediate associated with a negative charge on the acetyl oxygen atom, which is stabilized by two separate oxyanion holes (OAH). OAH1 is formed by Nε2 of His348 and a conserved catalytic water molecule and is involved in both steps of the catalytic cycle, while OAH2 is formed by peptide backbone nitrogen atoms of Cys89 and Gly380 and is only involved in the second step. The

catalytic water involved in forming OAH1 stabilized by a hydrogen bond to Asn316; an interaction shown to be essential for efficient catalysis [37].

The Claisen condensation reaction catalyzed by ACATs is a fundamental first step in the construction of carbon skeletal frameworks, essential for energy storage and normal cellular function in both prokaryotes and eukaryotes. There is also evidence to suggest that ACATs play a central role in cellular stress response. Bacterial ACAT catalyzes the first step in the synthesis of polyhydroxyalkanoates (PHA), the major energy and carbon storage molecules in many bacteria, important for survival under conditions of nutrient limitation or stress [25, 38]. Bacterial ACAT activity is inhibited by CoA; a negative feedback loop that is hypothesized to sense the metabolic state of the cell [39]. Soto *et al* [40] showed that expression of alfalfa cytosolic ACAT is upregulated under conditions of cold or salinity stress, and also that over-expression of alfalfa cytosolic ACAT in bacteria increased bacterial stress resistance. Importantly, they showed that plant ACAT is also inhibited by CoA. It therefore follows that isoprenoid production is dependent on intracellular CoA concentrations. CoA is released from the citric acid cycle under optimal growth conditions, but this pathway is inhibited under conditions of oxidative stress [39, 41-42]. It has therefore been suggested that, when CoA concentrations are high (during optimal conditions), thiolase activity is low and so the rate-limiting step is the third step in the pathway, catalyzed by HMG-CoA reductase (Figure 1); however, when CoA concentrations are low, thiolase activity is high and the first step, catalyzed by cytosolic ACAT, becomes the rate-limiting step [40]. Thus, it has been proposed that upon encountering conditions of stress, many organisms alter their metabolism from the TCA cycle to the PHA or mevalonate pathways to provide highly reduced molecules for the antioxidant response [40, 43]. In agreement with this, in eukaryotes, consumption of Ac-CoA for lipogenesis is inhibited when the intracellular ATP concentration is low (cAMP concentration is high) [44] – as would be the case when citric acid cycle is inhibited. Under these conditions, conversion of Ac-CoA to malonyl-CoA – the rate-limiting step in lipogenesis – is inhibited by AMP-activated protein kinase (AMPK). This would increase the Ac-CoA concentration, increasing thiolase activity in the synthetic direction. Indeed, inactivation of the AMPK homolog in yeast resulted in decreased cytosolic Ac-CoA concentrations, and reduced overall fitness and resistance to stress [45]. All of these observations suggest that cytosolic ACAT plays a key role in the cellular response to environmental stress in both prokaryotes and eukaryotes.

In fungi, *S. cerevisiae* cytosolic ACAT is the product of a single gene, *ERG10*, and is essential for viability in the absence of exogenous mevalonate [46]. Similarly, *Candida tropicalis* cytosolic ACAT null mutant is auxotrophic for mevalonate [20]. Unsurprisingly, *ERG10* has also been shown to be essential for growth in *A. fumigatus* [47], presenting cytosolic ACAT as a potential target for novel antifungals. Despite its importance for fungal cell growth and survival, there have been no structural studies on a fungal ACAT until now. Here, we present the X-ray crystal structure of cytosolic ACAT from *A. fumigatus* (afCT); representing the first structure of a fungal type II thiolase. To our surprise, afCT is more similar to human mitochondrial ACAT (hT2) than to human cytosolic ACAT (hCT) regarding its primary sequence, substrate binding pocket, accommodation of K^+ and Cl^- ions, and dependence on K^+ for activity. We have also investigated the enzyme's monovalent cation preference, with observations that challenge the current paradigm regarding activation of T2 by K^+ but not Na^+. Perhaps most importantly, our crystals have trapped several different catalytic site configurations, including both tetrahedral reaction intermediates of the thiolase reaction cycle, allowing us to clearly interpret the reaction mechanism of this enzyme for the first time in *A. fumigatus*, with implications for all members of the thiolase family.

EXPERIMENTAL SECTION

Reagents. Dithiothreitol was purchased from Apollo Scientific Ltd; all other reagents were purchased from Sigma-Aldrich™. CoA and AcAc-CoA stock concentrations were determined using Ellman's test [48] in 0.1 mM EDTA, 100 mM potassium phosphate pH 7.0. Protein purification columns were purchased from Bio-Rad Laboratories Pty., Ltd.

Sequence Analysis. A phylogenetic tree was generated by the "Simple Phylogeny" tool on the EMBL-EBI website using a Clustal omega multiple sequence alignment of ACATs from a range of species; The Interactive Tree of Life web-based tool [49] was used to generate Figure S1. Peroxisomal target signal sequences were predicted using the "Target Signal Predictor" web-based program [50]. Mitochondrial targeting peptide sequences were predicted using TargetP [51]. A Clustal omega multiple sequence alignment of select ACATs was uploaded to the ESPript 3.0 server [52] to produce Figure S2.

Protein Purification. The *A. fumigatus* cytosolic ACAT (afCT) DNA sequence (NCBI accession: XM_742114.1) was amplified from af293 cDNA (a generous gift from Dr. James Fraser, University of Queensland), cloned into pET-57-DEST via Gateway™ technology (Invitrogen™), and recombinant afCT was then over-expressed in a 3 L culture of *E. coli* BL21(λDE3) overnight at 16°C in shake-flasks. Cells were then resuspended in buffer (20 mM Tris pH 8.0, 0.5 M NaCl, 10% glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol) and lysed by mechanical disruption. Clarified cell lysate was subjected to Ni^{2+}-affinity chromatography twice, with a TEV digest in between to remove the hexahistidine and NusA tags. afCT protein was then purified to > 95% (as assessed by SDS-PAGE) by anion-exchange chromatography (using an ENrich™ Q 5 x 50 Column; Bio-Rad) and dialyzed overnight at 4°C to 10 mM NaCl, 1 mM DTT, 10 mM Tris pH 8.0. Protein was concentrated to approximately 8 mg/ml, flash-cooled in liquid nitrogen and stored at -80°C.

Enzyme Assays. The activity of the purified enzyme in the degradative direction was assessed *in vitro* at 25°C by the Mg^{2+} assay essentially as described [53] (Table 1). In the standard assay, the reaction was started by the addition of 300ng of afCT to 50 mM Tris-HCl pH 8.1, 20 mM $MgCl_2$, 42 µM CoA and 7.5 µM AcAc-CoA. The initial decrease in absorbance at 303 nm was then used to determine the rate of conversion of AcAc-CoA to Ac-CoA. The extinction coefficient for the Mg^{2+}-AcAc-CoA enolate complex under these conditions was determined to be 16000 $M^{-1}.cm^{-1}$, similar to that previously reported [29, 54]. The k_{cat} and apparent K_m for AcAc-CoA were determined by varying the concentration of AcAc-CoA from 0–38 µM. The addition of either NaCl or KCl substantially increased the reaction rate, with maximum activity achieved at approximately 100 mM KCl. Therefore, K_m and k_{cat} values were also determined in the presence of 100 mM NaCl or 100 mM KCl, with the addition of 200ng or 100ng afCT, respectively, to commence the reaction. To investigate the relationship between monovalent cation (MVC) ionic radius and activation of thiolase, chloride salts of other group I cations (Li, Rb and Cs), as well as ammonium, were also tested at 100 mM in the standard assay, with the addition of 200ng afCT to commence the reaction. All data were collected in triplicate, unless otherwise indicated, and fit in GraphPad Prism (v7).

Protein Crystallization. All protein crystallization experiments were performed at 16°C using the vapor-diffusion method. afCT apo crystals grew as plates in 20% polyethylene glycol 3350, 0.2 M KF to a maximum size of approximately 400x400 µm in 3 days in hanging drop format, with 4 µL drops (2 µL protein sample plus 2 µL reservoir solution) and 0.5 mL reservoir volume. In the same way, crystals of afCT in complex with rubidium or cesium were grown in 20% polyethylene glycol 3350, 0.15 M RbCl or 20% polyethylene glycol 3350, 0.15 M CsCl, respectively. Crystals of afCT in complex with substrate were obtained by the addition of 5 mM Ac-CoA to 8 mg/mL purified protein immediately prior to setting up crystallization screens. Crystals grew as approximately 200–300 µm rods after 3 days in two different conditions: 23% polyethylene glycol 3350, 0.2 M $(NH_4)_2SO_4$, 0.1 M Bis-Tris pH 6.5, 0.2 M KCl in hanging drop format, with 2 µL drops (1 µL protein plus 1 µL reservoir) and 0.5 mL reservoir volume; and 25% polyethylene glycol 3350, 0.2 M $(NH_4)_2SO_4$, 0.1 M Bis-Tris pH 6.5 in sitting drop format, with 2 µL drops and 75 µL reservoir volume

Data Collection and Refinement. Data collection and refinement statistics are presented in the Supporting Information in Table S1. Diffraction data were collected at 100K at the Australian Synchrotron (MX1 beamline) [55], using mother liquor supplemented with 16% glycerol as cryoprotectant. Each dataset consists of 360 frames collected at 1° intervals with 1° oscillation. All data were collected at an X-ray energy of 13000 eV, except for additional datasets collected at 8500 eV or 15350 eV for Cs^+ and Rb^+ containing crystals, respectively, to maximize anomalous signal. Datasets were processed using iMosflm [56] and structures were solved by molecular replacement (MR) using

phaserMR [57]. Human mitochondrial ACAT (hT2) (PDB code: 2IBY) was used as the initial search model for MR; the first afCT structure was then used as the search model for subsequent afCT structures. Atomic coordinates were refined using phenix.refine [58] using iterative cycles of rebuilding in Coot [59], with anisotropic B-factor refinement restricted to automatically defined torsion-liberation-screw (TLS) groups [60], and Rb^+ and Cs^+ ions individually, where applicable. Final models have good geometry statistics, with greater than 97% of peptide bonds within the favored region of the Ramachandran plot. The only Ramachandran outlier occurs at Val91 of all apo afCT structures, and subunit C of afCT+AcCoA+NH_4^+. Phenix.maps [61] was used to calculate anomalous difference maps, using data to 3.0 Å or 3.3 Å for afCT+Cs^+_{anom} and afCT+Rb^+_{anom} datasets, respectively. Anomalous difference Fourier maps for Rb^+ and Cs^+ ions, in addition to isomorphous difference maps derived from comparisons between data from crystals grown in the presence of KCl and those grown in the presence of RbCl or CsCl, were used to identify MVC binding sites. Atomic coordinates and structure factors for all models have been deposited in the RCSB Protein Data Bank (https://www.rcsb.org/) with the accession codes 6ARF, 6ARG, 6ARL, 6ARR, 6ART, 6AQP and 6ARE (see Table S1).

Molecular Docking. Docking of (2*S*)-methylacetoacetyl-CoA and (2*R*)-methylacetoacetyl-CoA separately to the substrate binding site of the D subunit of afCT+AcCoA+K^+ was performed manually, guided by the bound CoA molecule in the crystal structure. This was then followed by global optimization of side chains and annealing of the peptide backbone (20 iterations) using ICM Molsoft (v3.8-6a) [62].

Structure Visualization. Electrostatic surface representations were colored using electrostatic potential maps calculated by the APBS tool [63] in UCSF Chimera [64]. Atomic charges were first assigned using PDB2PQR [65]. All other molecular graphics images were produced using PyMOL(TM) (v1.8.6.0) [66]. All chemical diagrams were drawn using ChemDraw Ultra (v12.0).

RESULTS

Sequence Analysis. Protein sequences of animal, plant, fungal and bacterial thiolases were compared to provide insight into the evolution and function of *A. fumigatus* thiolases. A phylogenetic analysis can be found in Figure S1. *A. fumigatus* has genome annotations for four putative KATs and two putative ACATs [67]. Previous comparative genomic analyses of fungal species, along with *Aspergillus nidulans* gene deletion studies, have shown that, while yeasts such as *Saccharomyces spp.* and *Candida spp.* appear to have lost a mitochondrial β-oxidation pathway, retaining only a peroxisomal pathway, most fungi (including *Aspergillus spp.*) possess both mitochondrial and peroxisomal pathways [68-70]. Shen *et al* [70] showed that two of the four KATs in the *A. fumigatus* genome are likely localized to the mitochondria, and the other two to the mitochondria or peroxisome. Interestingly, all four KATs from *A. fumigatus* cluster with plant, yeast and human peroxisomal KATs, while human and fly mitochondrial KATs cluster separately. This suggests differences in the evolution of thiolase subcellular localization between lower eukaryotes and higher eukaryotes, and is also consistent with the fact that fungi lack a mitochondrial trifunctional enzyme (TFE) homolog, involved in the type II β-oxidation pathway in higher eukaryotes [70].

ACATs from all species cluster together, separate from the KATs. Both *A. fumigatus* ACAT sequences were analyzed for predicted mitochondrial targeting peptide (mTP) and peroxisomal targeting signal (PTS) sequences. One ACAT possesses neither a predicted PTS nor mTP, and the other contains an N-terminal mTP, consistent with their respective clustering with yeast and plant cytosolic ACATs or human and fly mitochondrial ACATs (Figure S1). *A. fumigatus* cytosolic ACAT is homologous to *S. cerevisiae* cytosolic ACAT (NCBI accession: NP_015297.1) – the product of the *ERG10* gene (NCBI Gene ID: 856079) [46]. Intriguingly, when compared to ACATs from higher eukaryotes, cytosolic ACATs from plant and fungi are more similar in terms of amino acid sequence to mitochondrial ACATs than to cytosolic ACATs. The crystal structure of human mitochondrial ACAT (hT2) revealed a K^+ binding site that stabilizes the substrate binding pocket and the catalytic histidine, providing a structural basis for its activation by K^+ ions [30]. In addition, a Cl^- ion was observed at the

dimer interface, also stabilizing active site residues. *A. fumigatus* cytosolic ACAT – referred to simply as cytosolic thiolase (afCT) herein – has a higher sequence identity to hT2 (49.6 %) than it does to human cytosolic ACAT (hCT) (44.3 %). Upon closer inspection of the multiple sequence alignment of fungal, human and bacterial ACATs (Figure S2), all the residues that differentiate hT2 from hCT (and bacterial ACAT (bCT)) with regards to Cl^- binding are conserved in afCT (Lys90, Asn385 and Gly389 with residue numbers referring to afCT), and most of the residues involved in coordination the K^+ in hT2 are well conserved in afCT (most notably, Tyr187). In addition, Phe325 of hT2, which has been suggested to be a key determinant for the broader substrate specificity of T2 over CT, is also conserved in afCT (Phe294). Interestingly, most of these residues are also conserved in yeast and *A. thaliana* cytosolic ACATs, consistent with the clustering of ACATs from fungi and plant with hT2, separately from hCT and bCT (Figure S1).

Enzyme Kinetics. Recombinant afCT was purified to homogeneity and assessed for AcAc-CoA thiolase activity in the degradative direction using the Mg^{2+} method (see Experimental Section). When the assay was performed in the standard protein buffer (low [salt]), thiolase activity was very low, with a turnover rate much lower than for mammalian and bacterial ACATs, and a higher K_m for AcAc-CoA (Table 1). The high sequence similarity between afCT and hT2, particularly in regions associated with ion binding, prompted us to determine whether the catalytic activity of afCT is modulated by K^+ ions in the same way as for hT2. afCT activity was markedly increased in the presence of KCl, with maximal activity achieved at a KCl concentration of approximately 100 mM. Addition of 100 mM KCl caused a 5-fold decrease in the K_m for AcAc-CoA and increased the k_{cat} by 14-fold. afCT catalytic efficiency was also enhanced in the presence of NaCl, addition of 100 mM NaCl resulted in the same decrease in K_m for AcAc-CoA and a 5-fold increase in k_{cat} to a value similar to the k_{cat} of hT2 in the presence of KCl, but still 3-fold lower than the k_{cat} of afCT in the presence of KCl (Table 1). This was unexpected, as it is currently accepted that T2 is activated by K^+, but not Na^+ ions [29-30]. Addition of 100 mM choline chloride resulted in no increase in afCT activity in the standard assay (Figure 3), showing that activation of afCT by NaCl is specific, and not simply a result of increasing ionic strength. It also rules out the possibility that activation is due to Cl^- ions rather than Na^+ ions. The MVC preference of afCT was investigated further by testing the effect of other monovalent cations (MVC) on afCT activity. Addition of chloride salts of all five common alkali-metal ions, or ammonium, increased the thiolytic reaction rate to some degree (Figure 3). Li^+ and Na^+ increased afCT activity 4-fold and 8-fold, whereas K^+, NH_4^+, Rb^+ and Cs^+ all increased afCT activity greater than 20-fold relative to low salt conditions, with the greatest activity observed in the presence of K^+ (34-fold increase). This suggests that afCT possesses a site(s) that accommodates MVCs with an optimal ionic radius of that of K^+ (1.38 Å, where coordination number = six), but is promiscuous for other MVCs also.

Thiolase Structure. X-ray crystal structures of afCT were solved by molecular replacement using data to resolutions of 1.70 to 2.25 Å. Structures were solved in apo and liganded forms in complex with different MVCs. Data processing and refinement statistics can be accessed in Supporting Information (Table S1). Occupancy refinement was performed for all ions, ligands, and residues with alternate conformations. For liganded structures, atoms of CoA were defined as a group separate from each individual acetyl group for refinement of group occupancies. Residues 1 to 398 could be confidently modelled into the electron density for all subunits in all structures (including Gly0 – the remnant of the TEV protease cleavage site), except for N-terminal residues 1 to 3 for subunits A of afCT+K^+, afCT+Rb^+ and afCT+Cs^+, and residues 211 to 214 of the cationic loop for most subunits across all structures. afCT crystallized in space group $P2_1$ in all cases, with four protein molecules per asymmetric unit, representing the biological tetramer (Figure 4A). The tetramer exhibits non-crystallographic 222 symmetry and is comprised of two tight dimers arranged head to head, with one dimer rotated approximately 45° with respect to the other, such that residues of the cationic loop (including basic residues Arg212 and Lys214) point towards the CoA binding pocket of the opposing subunit (Figure 4A). Each subunit is essentially identical (RMSD across all α-carbons is 0.15–0.29 Å) and is composed of an N-terminal domain (NTD), a loop domain and a C-terminal domain (CTD) (Figure S2 and 4B). Residues of Nβ2 and Nα2, including their extended connecting loop (Asn59 to

Asn68), along with Nβ3 and Nα3 of the NTD contribute most of the interface between tight dimers. Lβ1 and Lα1 of the loop domain and Cβ1 of the CTD make up the remaining interface surface. The interface surface areas between directly opposite subunits (i.e. A and C or B and D) and diagonally opposite subunits (i.e. A and D or B and C) are approximately 344 Å^2 and 758 Å^2, respectively. This is significantly less than the dimer interface surface area (i.e. between A and B or C and D), which is approximately 2494 Å^2. For all afCT structures, the average B-factor of one dimer is approximately double that of the opposing dimer (see Table S1), indicating that the weaker tetramerization interaction allows for a degree of flexibility between the two tight dimers. This has been observed previously for other tetrameric thiolases [24, 36, 71], but its functional significance remains unknown.

The cage-like tetramerization motif is typical of biosynthetic thiolases, and has been described in detail previously [24]. It is composed of residues His127 to Met148 of the loop domain (Figure S2). This loop extends away from the subunit core, allowing Tyr138, at the distal end of the loop, to form part of the entrance to the pantetheine binding tunnel leading to the thiolase active site of the diagonally opposite subunit, and fill a hydrophobic pocket on its surface formed by Ile147, Met148, Leu152 and Leu255. Lβ1 (residues 127-129) and Lβ1' (residues 141-144) form an antiparallel β-sheet that is partially continuous with the equivalent β-sheet of both its dimeric partner, and of the subunit diagonally opposite, such that a small distorted β-barrel is formed. Twelve leucine residues; Leu129, Leu132, and Leu143 from each subunit, point inwardly to form the hydrophobic core of the cage. Four salt-bridges formed between Arg133 and Asp145 of dimeric partners at the top and bottom of the cage stabilize the structure. Also contained within the loop domain is the cationic loop (residues 203-221), which extends into the inter-tetramer space towards the substrate binding site of the opposing subunit (Figure 4). A highly conserved arginine at the N-terminus (Arg14) appears to be responsible for anchoring the cationic loop to the NTD via electrostatic interactions with highly conserved acidic residues at either end of the cationic loop sequence (Glu202 and Asp222) (see Figure S2). The importance of these interactions was highlighted by a deletion mutation of the residue preceding Asp222 identified in hT2 from a T2-deficient patient [31].

The NTD and CTD share a βαβαβαββ topology, with the loop domain inserted in between the final two β-strands of the NTD; a secondary structural topology well conserved among thiolases [19, 24]. Each terminal domain is composed of a central mixed β-sheet, flanked by helices on either side. These domains are rotated approximately 180° with respect to one another, and pack face to face such that Nα3 and Cα3 are at the center, sandwiched between the β-sheets (Figure 4B).

Comparison to ACATs from other Species. The afCT monomer has a tertiary structure very similar to hCT, hT2 and *Z. ramigera* ACAT (bCT), with average RMSD values across all C-alphas of 1.20–1.34 Å, respectively (Figure S3A). Superposition of dimers gives similar RMSD values (1.20–1.37 Å). Inclusion of the whole tetramer in this superposition demonstrates a degree of variation regarding the relative position of the second dimer (Figure S3B). This signifies a departure from exact 222 symmetry, consistent with the flexible nature of the tetramerization motif, and has been discussed in detail previously [71].

The main differences between afCT and the other cytosolic ACATs (hCT and bCT) concern residues at the dimerization interface and substrate binding pocket, corresponding to regions that differentiate hCT from hT2 and have been shown to be involved in binding of K^+ and Cl^- ions in hT2 [30].

Binding of Monovalent Cations near the Substrate Binding Pocket. To probe for MVC binding sites, crystals of afCT were grown in the presence of K^+, Rb^+ or Cs^+ ions. Anomalous difference Fourier maps for Rb^+ and Cs^+ ions (Figure S4), in addition to isomorphous difference maps derived from comparisons between data from crystals grown in KCl and those grown in RbCl or CsCl, were used to identify MVC binding sites. Average anomalous difference map peak heights are listed in Table 2 (Tables S2 and S3 list anomalous and F_o-F_o peak heights for all Cs^+ and Rb^+ sites, respectively). 13 binding sites were identified for Rb^+, and 15 for Cs^+. All of the Rb^+ binding sites correspond to Cs^+ binding sites, with the two extra Cs^+ ions present at low occupancy (approx. 50%) at sites distant from the active site, implying that they are likely to be crystallization artefacts. Of the 13 MVC sites common

to the Rb^+ and Cs^+ structures, one of these is part of a crystal contact, consistently present at low occupancy (approx. 50%), indicating that it is also likely to be a crystallization artefact.

Based on these results, 13 K^+ ions were modelled at the sites common to the Rb^+ and Cs^+ structures, in both the apo (afCT+K^+) and liganded (afCT+AcCoA+K^+) structures for which the crystallization conditions contained K^+. The K^+ ions are in the same position in each subunit of the tetramer and form similar coordination bonding. For the latter structure, K^+ modelled into MVC binding sites refined to occupancies of between 43 and 97%. The crystallization condition for this structure contained equal concentrations of K^+ and NH_4^+ ions, therefore, given the high activity of afCT observed in the presence of K^+ or NH_4^+, and their similar ionic radii (1.38 Å and 1.48 Å, respectively), it is likely that they would compete for the same binding sites, explaining the lower occupancy of K^+ at MVC sites in this structure (Table S4). In addition, a structure of liganded afCT in the presence of only NH_4^+ (afCT+Ac-CoA+NH_4^+) was obtained, for which NH_4^+ ions were modelled in all cation binding sites for which there was appropriate electron density (10 sites). Disregarding the MVC site that is part of a crystal contact, each subunit has the same three MVC binding sites (Figure 4). Two are positioned at the C-terminal ends of separate surface exposed helices and coordinated by three or four water molecules and three carbonyl oxygens of adjacent residues within surface-exposed loops. The other is situated near the adenosine binding pocket (Figure 5). Based on proximity to the substrate binding pocket, coordination by protein atoms, and average occupancy and B-factors for Cs^+, Rb^+, K^+ and NH_4^+ in all structures (see Tables S2-S4), the latter of these three binding sites is most likely to be relevant to the modulation of thiolase activity *in vivo*.

This site is homologous to the K^+ binding site of hT2 [30], with approximate octahedral coordination by six oxygen atoms. The K^+ is coordinated by one water molecule (Wat_{MVC}) (2.7 Å) and five protein atoms: the backbone carbonyl oxygens of Ala249 (3.3 Å), Pro250 (2.7 Å), Ser252 (2.6 Å) and Val350 (2.8 Å) along with the hydroxyl oxygen of Tyr187 (2.8 Å) (Figure 5). Ion coordination at this site is essentially identical in liganded and apo afCT structures, aside from a small shift in Wat_{MVC} (~0.7 Å), facilitating the formation of a hydrogen bond (3.0 Å) to the adenine base (N7) of CoA in the liganded structure. Similarly, K^+ coordination at this site is essentially identical for afCT and hT2, with the only significant difference being a 0.6 Å shift of the coordinated water closer to the position of the bound CoA molecule, caused by the substitution of Pro254 (afCT) for Thr285 (hT2) (Figure S5). It has been previously suggested that, for hT2, coordination of a K^+ ion at this position helps to rigidify the adenine binding pocket and stabilize the Cβ3-Cα3 loop, which contains the catalytic histidine [30]. Ala249, Pro250, Ser252 and Tyr187 of afCT all make direct contact with bound CoA substrate and are contained within regions of the loop domain that contribute most of the residues involved in substrate binding. Val350 is part of a short helix (Cα2') immediately preceding the loop containing the catalytic histidine, His354 (Figure 5).

The mean length of coordination bonds (across all subunits) for K^+, Rb^+ and Cs^+ ions bound at this position are 2.86 Å, 2.96 Å and 3.12 Å, respectively. These values correspond very well to mean six-coordinate M^+--O^{2-} complex bond lengths determined previously of 2.828 Å, 2.989 Å and 3.124 Å, respectively [72]. Superposition of these three apo structures shows that substitution of K^+ for Rb^+ or Cs^+ has no effect on the position of any of the coordinating atoms except for Wat_{MVC}, which shifts away from the MVC as the radius of the ion increases (Figure S6). This increases the distance between the relative positions of Wat_{MVC} in apo and liganded structures. For example, in the Cs^+-bound structure, Wat_{MVC} is shifted 2.0 Å from the position it occupies when forming a hydrogen bond with the adenine (N7) in the liganded structure. K^+ sits in the same plane as the coordinating oxygens of Tyr187, Ala249, Ser252 and Val350; however, due to their size, both Rb^+ and Cs^+ shift out of this plane, such that Cs^+ is positioned 0.5 Å closer to the substrate binding site. This less ideal coordination geometry, accompanied by the shift of Wat_{MVC} away from its substrate-interacting position, provides a molecular explanation for why K^+ is better able to stimulate afCT activity than these larger MVCs. Conversely, the planar geometry of K^+ with four coordinating ligands suggests that the site is larger than ideal for smaller MVCs; Octahedral Li^+--O^{2-} and Na^+--O^{2-} complexes have average bond lengths of 2.178 Å and

2.441 Å, respectively [72]. Therefore, although the MVC site of afCT can accommodate all common alkali-metal ions, its size is ideal for coordination of K^+.

Calculation of an electrostatic potential surface map shows a strong negative charge at the MVC binding pocket, largely contributed by Glu224 (Figure S7A). In the absence of MVCs, electrostatic repulsion would exist between this negatively charged region and the pyrophosphate of CoA. MVC binding would increase the positive electrostatic potential of the adenosine binding pocket, increasing its affinity for the negatively charged phosphate groups of CoA. For hCT, a water molecule occupies a similar position to the K^+ of afCT and hT2 (Figure S7C). This water molecule is coordinated by only five oxygens: three backbone carbonyl oxygens (corresponding to Ala249, Ser252 and Val350 of afCT), and two other waters. The major differences between afCT and hCT regarding this pocket are the substitution of Tyr187 for Gln186 and Glu224 for Phe221. The significance of Tyr187, which provides a K^+ coordinating oxygen, is highlighted by its conservation across fungal ACATs and hT2 (Figure S2). A glutamine is present at this position for both hCT and bCT, which is too far from the K^+ site to donate a coordinating oxygen. Glu224, which carries the closest negative charge to the K^+ (4.4 Å), is conserved in hT2, but substituted for a phenylalanine or tyrosine in hCT or bCT, respectively, decreasing the negative electrostatic potential of the pocket (Figure S7B).

Chloride Ion Binding Site. Residues that were identified to make contacts with the Cl^- ion in hT2 are conserved in afCT (Figure S2). This prompted us to model Cl^- ions at these sites for afCT. In addition, small anomalous difference peaks for data collected at 8500 eV (Table S1, afCT+Cs^+_{anom} dataset) provided further validation that these Cl^- ions were modelled correctly (Figure 6). There are two identical Cl^- binding sites at each dimer interface (one for every subunit), within approximately 10 Å of the thiolase active site (Figure 6). The Cl^- is positioned between two basic residues – Arg71 from one monomer, and Lys90 from the dimeric partner – and is coordinated by five atoms: Arg71 (Nε) (3.5 Å), Asn385 (Nδ2) (3.2 Å), Gly389 (N) (3.3 Å), Wat_{CL1} (2.6 Å) and Wat_{CL2} (2.7 Å), consistent with a recent survey of PDB structures indicating that five is the preferred coordination number for Cl^- within proteins [73]. Even though it is not involved in catalysis directly, this Cl^- stabilizes the catalytic site via both direct and water-mediated hydrogen bonds to catalytic residues and residues adjacent. Aside from forming a coordination bond with Asn385 – which is flanked on either side by catalytic residues, Cys384 and Gly386 – the Cl^- interacts with Gly386(O) via Wat_{CL1}, and Lys90(Nζ) via Wat_{CL2} and Wat_{CL3}. Comparison with the structure of hT2 shows that Ala102 and P118 of hT2 are substituted for Asn68 and Ile84 in afCT, respectively (Figure S8A). The effect of these two substitutions is to widen the afCT Cl^- binding pocket slightly, allowing one extra water molecule as compared to hT2, and shifting the Cl^- by 1.6 Å, so that it is closer to Arg71 and the Cβ4-Cβ5 active site loop (containing Cys384, Asn385 and Gly386). In addition, the afCT site much more solvent accessible than the hT2 site. The Cl^- is connected to bulk solvent via a chain of waters leading to Gly156 on the surface of the protein. For hT2, this residue is Tyr188, which disrupts this chain of waters. Interestingly, of all the ACAT sequences compared in Figure S2, afCT is the only one without an aromatic residue at this position.

Regarding the region corresponding to the Cl^- binding site, the dimerization interfaces of hCT and bCT are much more hydrophobic compared to afCT and hT2. Although Arg71 is highly conserved, Lys90, Asn385 and Gly389 are all substituted for hydrophobic residues: Met90, Ile384 and Met388 in hCT, with the latter of these occupying the space of the Cl^- and Wat_{CL2} in the afCT structure (Figure S8B).

Mode of CoA Binding. In addition to forming the tetramerization motif, the loop domain contains most of the residues that are important for substrate binding. Average B-factors for protein atoms of liganded structures are much lower than for the apo structures (Table S1), indicating that substrate binding stabilizes the polypeptide. The CoA binding site can be conceptually divided into two pockets: the 3'-phosphoadenosine binding pocket and the pantetheine binding pocket. Both pockets open into the inter-tetramer space. Loop domain residues 224 to 237 and 249 to 250, along with Tyr187, form the 3'-phosphoadenosine binding pocket (Figure 7A). Of note are five basic residues within 10 Å of the phosphate groups of CoA: Lys232 and Lys237 of the subunit in question, Lys137 of the

diagonally opposite subunit, and Arg212 and Lys214 of the cationic loop (residues 203-221), which extends into the inter-tetramer space from the opposing subunit (note that the sidechains of Arg212 and Lys214 are unstructured in almost all structures; however, the proximity of the peptide backbone affords the possibility that they interact with the 3'-phosphate of CoA). Similar positioning of basic residues in tetrameric ACATs from other species has been suggested to facilitate electrostatic interactions with CoA to aid substrate capture [24, 74]. One of these basic residues – Lys232 – forms a salt bridge with the 3'-phosphate of CoA. Despite possessing numerous hydrogen bond donors and acceptors, there are only three other hydrogen bonds made directly between CoA and protein atoms: Tyr187 (OH) to adenosine (N6) (2.9 Å), Asn229 (N) to adenosine (N1) (3.2 Å) and Ser253 (O) to pantetheine (N8) (2.8 Å) (Figure 7B). All other hydrogen bonds are mediated by surrounding water molecules, including the Wat_{MVC}-mediated interaction with K^+, mentioned previously. A similar lack of direct bonding has been noted previously for bCT, which possesses only one direct hydrogen bond between protein and substrate [24]. All residues that interact with bound substrate are from the same subunit, with the exception of Tyr138, which protrudes from the tetramerization loop of the diagonally opposite subunit, forming part of the entrance of the pantetheine binding tunnel and participating in a water-mediated hydrogen bond with pantetheine (O9) (Figures 7A and B). Interestingly, of the ACAT sequences compared in Figure S2, this tyrosine is only conserved in hT2. Ala249 and Pro250, which both provide oxygen atoms for coordination of the K^+, contact the adenosine of CoA. The pyrophosphate is less than 3.5 Å from Ala249, such that the CoA molecule bends around Ala249 at this point. Pro250 packs against the adenine rings (< 3.7 Å). Leu233 and Ile236 also contribute van der Waals interactions with the adenine and ribose groups, respectively. For hT2, similar van der Waals interactions are facilitated by corresponding residues: Ala280, Ala281, Val264 and Leu267. Residues 227 to 230 of the Lβ3-Lα3 loop, which wrap around the bound adenine in afCT, are shifted approximately 2 Å away from the interdimer space relative to the same region of hT2 (Figure S9A). This is largely caused by the substitution of Phe261 in hT2 for Pro230 in afCT, and results in a similar small shift in the adenosine (~1 Å) deeper into the protein. Despite this, the overall structure of the adenosine binding pocket and key interactions are highly conserved between afCT and hT2.

Unlike hT2, the adenine binding pocket is much less conserved between afCT and other cytosolic ACATs. hCT and bCT contain a small uncharged residue (Ala230 of hCT) at the position corresponding to Lys232 of afCT, and therefore lack this salt-bridge to the 3'phosphate of the adenosine. Both also contain one extra residue in the Lβ3-Lα3 loop (220-229 of afCT) – His224 of hCT and His221 of bCT – which packs down against the surface of the protein away from the bound CoA, resulting in a significant rearrangement of this loop relative to the afCT structure (Figure S9B). This allows the preceding arginine (hCT Arg223, bCT Arg220) to stack against the adenine rings of CoA – an interaction that is absent from both afCT and hT2 structures.

Aside from Ser252 and Ser253, the pantetheine pocket leading to the catalytic site is lined exclusively by hydrophobic residues: Met122, Leu152, Met161, Gly162, Ala239, Phe240, Pro254 and Leu255 of the loop domain, and Phe294, Ala324, Phe325 and Ile356 of the CTD. These residues are highly conserved across fungal, bacterial, human and plant ACATs (Figure S2), with the notable exceptions of Ala239, Pro254 and Phe294. Also, Leu160, which is positioned approximately 7 Å away from the pantetheine at the tunnel entrance, is substituted for a histidine in hT2 (His192), hCT (His159) and bCT (His156) which contacts the pantetheine of the bound substrate (Figure S9A and B). The area occupied by this histidine is replaced by a water molecule in afCT, mediating a hydrogen bonding interaction between pantetheine (O5) and Gly162 (N) (Figure 7B). Ala239 is conserved for bCT, but substituted for Tyr237 in hCT (Figure S9B). This tyrosine provides more surface area for van der Waals interaction with the pantetheine moiety, but does not appear to significantly alter the relative position of the bound substrate. Pro254 is near the K^+ binding site, and is substituted for a glycine in both hCT and bCT, probably to allow for the extra bulk of the aromatic residue that occupies the space of Glu224 near the K^+ site mentioned earlier.

Accommodation of 2-methylacetoacetyl-CoA by afCT. In addition to catalyzing the synthesis and degradation of AcAc-CoA, T2 catalyzes the last reaction in the isoleucine degradation

pathway, where 2-methylacetoacetyl-CoA is converted to propionyl-CoA and Ac-CoA in the mitochondria [27]. Human T2 deficiency results in accumulation of isoleucine degradation metabolites. Substitution of afCT Phe294 for methionine in both hCT and bCT is the only major difference between these enzymes at their catalytic sites. Phe294 is conserved in hT2 (Phe325), and has been linked to the broader substrate specificity of hT2 compared to hCT. The Phe325-Pro326 dipeptide of hT2 expands the catalytic cavity relative to hCT (for which the corresponding sequence is Met293-Gly294), allowing it to accommodate the larger branched chain substrate, 2-methylacetoacetyl-CoA [30]. bCT has been shown to have no activity towards 2-methylacetoacetyl-CoA [25]. Strikingly, Phe294 is conserved across all ACAT sequences compared in Figure S2, except for hCT and bCT, for which it is substituted for a methionine. Specifically, the Phe-Pro dipeptide is conserved in both mitochondrial ACATs (*A. fumigatus* mACAT and hT2); both hCT and bCT contain Met-Gly; all other cytosolic ACATs contain a Phe-Thr dipeptide at this position. In addition, afCT Asn332 is conserved across all these ACATs except for hCT and bCT, which both contain an aliphatic residue at this position (Figure S2). The Nδ2 atom of Asn332 (hT2 Asn363) forms a hydrogen bond (2.9 Å) with the backbone O atom of Thr295 (hT2 Pro326), and is possibly important for anchoring the preceding phenylalanine to allow room for the extra methyl group of this branched chain substrate.

A simple molecular docking experiment suggests that afCT can indeed accommodate 2-methylacetoacetyl-CoA, requiring only a minor (20°) rotation of Phe294 away from substrate. This places Phe294 in an almost identical position to that observed for the corresponding phenylalanine (Phe325) in the structure of hT2 in complex with CoA (PDB: 2IBY) [30]. The extra methyl group is in van der Waals contact with the phenyl ring of Phe294 and the thioester and 3-keto O atoms are positioned in OAHs 1 and 2, respectively (Figure S10). This is consistent with the results of a similar docking experiment performed for hT2 [30]. These results potentially implicate afCT in isoleucine metabolism; however, further investigation is needed to confirm this and clarify its biological significance.

Catalytic Site. The catalytic residues – Cys92, His354, Cys384 and Gly386 – are contained in three loops: Nβ3-Nα3, Cβ3-Cα3 and Cβ4-Cβ5, in the interior of each subunit (Figure 8). Conserved glycines (95, 357 and 360) of the two central helices (Nα3 and Cα3) allow them to pack very close to one another, positioning the catalytic site at the N-terminal ends of two long helices, taking advantage of helix dipoles to decrease the pKa of active site cysteines and assist with stabilization of negatively charged species formed during the thiolase catalytic cycle (Figure 2) [75]. In addition, Asn322, which is part of the signature NEAF motif of thiolases located in the Cβ2-Cα2 loop (corresponding to Asn316 of bCT), stabilizes a catalytic water (Wat_{cat1}) via a hydrogen bond. Wat_{cat1} and His354 (Nε2) form OAH1. The peptide backbone nitrogens of Cys92 and Gly386 form OAH2. Cys92, which serves as the nucleophile in the first acetyl-transfer (Figure 2, stage 2), is contained within a 3_{10}-helix at the N-terminal end of Nα3; a strained conformation observed previously for other thiolases [36, 74], stabilized by hydrogen bonds between Ser94 (OH) and the peptide backbone of Lys90 and Val91. Val91 is the only Ramachandran outlier in any of the structures. It is only present consistently in afCT apo structures, suggesting that this strained peptide conformation is 'relieved' during the thiolase reaction cycle.

For both liganded structures (afCT+AcCoA+K^+ and afCT+AcCoA+NH_4^+), CoA could be modelled into all active sites with an occupancy of 76% or greater. Ligand binding induces no large conformational changes in protein structure; RMSD values between apo and liganded afCT are 0.24 Å or 0.67 Å across Cαs of a single subunit or the entire tetramer, respectively. The only notable changes occur at the active site, regarding the side chain conformations of Met161 and Cys92 (Figure 8). Sδ of Met161 is shifted 2.6 Å away from the active site center upon ligand binding to allow room for the pantetheine of CoA. Binding of CoA also induces a small shift in the position of Cys92 (Sγ), 1.4 Å towards the sidechain of His354. It is postulated that this shift facilitates the deprotonation of Cys92 (Sγ) by His354 (Nε2), allowing Cys92 to act as the nucleophile in the first acetyl transfer [35]. Once bound, the sulfur of CoA is then positioned only 4.1 Å away from Met161 (Sδ), 4.0 Å from Cys92 (Sγ), and 5.4 Å from Cys384 (Sγ). Positioning of CoA (S) near sulfur atoms at the thiolase active site is important for high affinity binding of substrate [76].

Approximately 15 ordered water molecules are displaced by substrate binding; nine from the adenosine pocket and six from the pantetheine pocket. In addition, there is a chain of three water molecules that begins at the active site and penetrates further into the interior of the protein. This includes Wat_{cat1}, which, along with His354 (Nε2), forms OAH1. In contrast, for both hCT and bCT this chain of waters includes approximately ten waters forming a hydrogen-bonding network that extends through a narrow cavity between Cβ2 and Cα2 through to solvent at the backside of the protein [35, 74]. It has been suggested that this water network may modulate the electrostatic environment of the catalytic site, promoting catalysis, and may explain the lower catalytic efficiency of T2 (which lacks this network) compared to hCT and bCT [37]. In both afCT and hT2 structures, only the first two or three waters of the network are present, closest to the catalytic site. Comparison of the sequences and crystal structures of afCT and hT2 with hCT and bCT indicates that substitution of several residues within Cα2 may account for the narrowing of this cavity, disallowing the presence of water molecules (Figure S11). Ser326, Val327 and Leu330 of afCT pack against Lα1 and Lα2, and are all larger than the equivalent residues of hCT, which are all alanine residues. These three Cα2 residues are on the side of the helix opposite to Cβ2, shifting the helix closer to Cβ2. Another Cα2 residue, Asn332, is conserved in all ACATs except hCT and bCT (where it is substituted for an aliphatic residue; Figure S2) and forms hydrogen bonds with backbone atoms of Cα1 (Thr295 and Ala299), also drawing Cα2 closer to Cβ2 (Figure S11). In addition, Tyr182 occupies what would be the entrance to the cavity at the surface of the protein.

Although both liganded structures share similar global folds (Cα-RMSD of tetramers = 0.66 Å), notable differences in the electron density present in the catalytic sites of the enzyme prompted us to model the atomic composition at each active site of the tetramer in both structures separately using Polder Maps to both reduce model bias and enhance weak density that may be masked by bulk solvent [77]. The observed density indicates that Cys92 is modified such that OAH2 is occupied, and at least one acetyl group is present in every active site. Given that CoA occupies every active site, and there is density for at least one acetyl group, there are seven different configurations of atoms possible, corresponding to stages two through eight of the reaction cycle shown in Figure 2. The electron density suggests that we have trapped multiple reaction intermediates.

For five of the eight active sites, CoA and an acetylated Cys92 (Ac-Cys92) corresponded to the electron density (Figure 9A), with the occupancy of the acetyl group between 63 and 76%, in agreement with the occupancy of the corresponding bound CoA molecule. OAH2 is occupied by the acetyl carbonyl oxygen, with hydrogen bonds to Cys92 (N) (2.9 Å) and Gly386 (N) (2.4 Å). This represents stage 4 of the reaction cycle, after acetyl-transfer from the first Ac-CoA to Cys92. For biosynthetic thiolase, the rate-limiting steps in both biosynthetic and degradative directions are those involving deacetylation of the active site cysteine [35], therefore it is unsurprising that this is the most commonly observed active site configuration. In all these cases there is additional density observed in OAH1. This space is occupied by a water molecule in the apo structure (afCT+K^+), in which the second water is absent from the water chain that begins with Wat_{cat1} (Figure 8). This suggests that Wat_{cat1} shifts forward into OAH1, accompanied by a shift in the adjacent water (Wat_{cat2}) from position 2 in the water chain to the original position of Wat_{cat1} (position 1), leaving position 2 vacant, as observed for the apo structure. To reflect this, for the liganded structure, a water molecule was modeled with two alternate conformations: (1) in position 2 of the water chain (Wat_{cat2}) or (2) occupying OAH1 (Figures 8 and 9A); Subsequent refinement resulted in occupancies of approximately 50% at both positions. The position of the thioester O atom of Ac-Cys92 (occupying OAH2) that we observe conflicts with previous structural studies on bCT [24, 35-36]. This is discussed further in the subsequent section.

Additional electron density present between the sulfur atoms of CoA and Cys92 in one of the afCT+AcCoA+K^+ active sites promoted us to model a second acetyl group, as Ac-CoA (Figure 9B). This clashed with the Ac-Cys92 and therefore was modelled as an alternate conformation, corresponding simultaneously to stages 2 and 4 of the reaction cycle (Figure 2) – immediately before and after the first acetyl-transfer. For stage 2, the thioester O of Ac-CoA occupies OAH1, with hydrogen bonds to Wat_{cat1} (2.4 Å) and His354 (Nε2) (2.6 Å). This stabilizes the partial negative charge present

on this oxygen, increasing the electrophilicity of the adjacent C1 atom. Cys92 (Sγ) is very close to C1 of the acetyl group (2.3 Å), poised for nucleophilic attack. For stage 4, the carbonyl O of Ac-Cys92 occupies OAH2 in the same way as for the active sites with CoA and Ac-Cys92 described above.

Tetrahedral Intermediates Trapped for both Acetyl-transfer Reactions. For a single active site of the afCT+AcCoA+NH_4^+ structure, there is strong density connecting C1 of Ac-Cys92 to the sulfur of CoA. The shape of the density indicates that Cys92 (Sγ), the acetyl group, and CoA (S) adopt a tetrahedral geometry (Figure 10A). Therefore, covalent bonds were defined from the acetyl group C1 atom to both Cys92 (Sγ) and CoA (S). This represents the tetrahedral intermediate formed during acetyl-transfer from Ac-CoA to Cys92 (Figure 2, stage 3). The negatively charged thioester O atom is positioned in OAH2, stabilized by hydrogen bonds to Cys92 (N) (3.2 Å) and Gly386 (N) (2.6 Å). While the conformation of this Cys92-Ac-CoA tetrahedral intermediate is well justified by the electron density, it is inconsistent with the previous suggestion that the first acetyl transfer proceeds via a tetrahedral intermediate stabilized by OAH1 [36]. No density is observed in OAH1, suggesting that the S atom of CoA – which is approximately 0.6 Å closer to Cys92 (Sγ) than for the active sites containing CoA and Ac-Cys92 – prevents Wat_{cat1} from shifting into OAH1, as observed for the CoA + Ac-Cys92 active sites (Figure 9A).

For the remaining active site of afCT+AcCoA+NH_4^+ there is continuous density occupying both OAHs and connecting CoA (S) to Cys92 (Sγ). This was explained well by modelling an acetyl group in OAH2 covalently linked via C1 to both Cys92 (Sγ) and C2 of a bound Ac-CoA molecule (Figure 10B). This configuration represents the tetrahedral intermediate that occurs during the second acetyl-transfer – from Cys92 to Ac-CoA – to form AcAc-CoA (Figure 2, stage 7). The negative charge present at the thioester O atom of the first acetyl group is stabilized by OAH2, with hydrogen bonds of 2.5 Å and 2.8 Å to Cys92 (N) and Gly386 (N), respectively. The thioester O atom of Ac-CoA partially occupies OAH1, but is further away from His354 (Nε2) (3.8 Å) and closer to Wat_{cat1} (2.2 Å) compared to its position immediately prior to the first acetyl-transfer (stage 2) shown in Figure 9B. Superposition of this catalytic site with the structure of bCT in complex with AcAc-CoA (the active site cysteine – Cys89 – was mutated to serine) (PDB: 1M1O) [36] shows that the acetyl groups of the tetrahedral intermediate occupy similar positions as the reaction product, AcAc-CoA, as expected (Figure 10B).

DISCUSSION

Implications for the Thiolase Catalytic Cycle. Crystal structures representing all five different acetyl-group configurations of the catalytic site have been determined previously for *Z. ramigera* ACAT (bCT) (stages 1 [35], 2 [36], 4 [24], 5 [35] and 8 [36] of the reaction cycle shown in Figure 2); however, a crystal structure of an ACAT in complex with a tetrahedral reaction intermediate (Figure 2; stages 3 or 7) has not been reported until now. Here we present structures of *A. fumigatus* cytosolic ACAT (afCT) in complex with both tetrahedral reaction intermediates previously predicted to form during each of the acetyl-transfer reactions. Different active sites of the crystallized tetrameric enzyme trapped substrates/intermediates at different stages of the reaction cycle depicted in Figure 2, including: (1) Ac-Cys92 + CoA (stage 4); (2) a site containing alternate conformations of Cys92 + Ac-CoA as well as Ac-Cys92 + CoA (stages 2 and 4, respectively); (3) Cys92-Ac-CoA tetrahedral intermediate (stage 3) and (4) Cys92-Ac-Ac-CoA tetrahedral intermediate (stage 7). These final two structures complete the structural repertoire of all stable intermediates that occur during the thiolase reaction cycle, allowing us to observe with direct, experimental structural evidence the full reaction mechanism for the first time.

Regarding the first acetyl-transfer reaction (Figure 2; stages 1 to 4), our structural data shows that Ac-CoA binds with the thioester O atom occupying OAH1 (Figure 9B), and that upon nucleophilic attack of Cys92 (Sγ) on the electrophilic C1 atom, the acetyl group rotates into OAH2 where the negative charge on the thioester O atom of the tetrahedral intermediate is stabilized by hydrogen bonds to the backbone N atoms of Cys92 and Gly386 (Figure 10A). After acetyl-transfer, the thioester O of

Ac-Cys92 remains in OAH2 (Figure 9A). This sequence of events conflicts with previous studies that have suggested that, during acetyl-transfer, the thioester oxygen atom of the acetyl group occupies OAH1, and only shifts to OAH2 upon binding of a second Ac-CoA [36]. This previous conclusion was based on the interpretation of four structures of bCT: (1) a C89A mutant bCT in complex with Ac-CoA (stage 2) showing that the acetyl group binds in OAH1 (PDB: 1M3Z) [36], (2) the acetylated enzyme (Ac-Cys89) in complex with CoA (stage 4) showing that the thioester O atom points away from OAH2 and is positioned between CoA (S) and Cys378 (O) (PDB: 1QFL) [24], (3) the unliganded acetylated enzyme (between stages 4 and 5) showing that the thioester O atom partially occupies OAH1 while the C2 atom occupies OAH2 (PDB: 1M4S) [36] , and (4) the acetylated enzyme in complex with Ac-CoA (stage 5) showing that binding of the second Ac-CoA shifts the thioester O atom of Ac-Cys89 into OAH2 (PDB: 1DM3) [35]. Of these structures, (1) and (4) are consistent with our interpretation of afCT structures. We suggest that the electron density observed at the active sites of bCT structures (2) and (3) was insufficient to accurately interpret the orientation of the acetyl group. Therefore, based on the structures presented herein, for which there is strong, readily interpretable density, we propose that the tetrahedral intermediate formed during the first acetyl-transfer reaction is stabilized by OAH2, as indicated by the reaction scheme shown in Figure 2 (stage 3).

Regarding the Claisen condensation reaction represented by the second tetrahedral intermediate (Figure 10B), the nucleophilic character of C2 of the second acetyl group (Ac-CoA) is imparted by proton abstraction by Cys384 (Sγ) [34]. This C2 is 4.8 Å from Cys384 (Sγ); too far for proton abstraction, suggesting that proton abstraction and nucleophilic attack are discrete steps, consistent with previous biochemical studies indicating that C-H bond cleavage is separate from C-C bond formation [25, 34] and that OAH1 is critical for the stabilization of the enolate ion formed after proton abstraction has occurred [37] (Figure 2 – stage 6).

Enzyme Activation by MVCs. We have shown that, like hT2, the catalytic efficiency of afCT is greatly increased by K^+. Numerous examples of MVC-activated enzymes have been documented; all those characterized thus far have a preference for either Na^+ or K^+ [78-80]. Na^+-activated enzymes, such as thrombin [81] and β-galactosidase, are generally poorly activated by smaller (Li^+) or larger (K^+, Rb^+, Cs^+) MVCs. K^+-activated enzymes, such as kinases [82-84] and molecular chaperones [85], are generally also activated partially by NH_4^+ and Rb^+, but not by Li^+, Na^+ or Cs^+ [78]. MVC-activated enzymes are classified into two broad categories [79]. Type I MVC-activation involves direct contact between the MVC and substrate, and the requirement for MVCs is usually absolute. This is typified by enzymes involved in phosphoryl transfer reactions, such as kinases, which require both a divalent metal ion and a MVC. The MVC is required for ATP binding and polarization of phosphate groups, and sometimes stabilization of the active conformation of the protein. Type II activated enzymes have a basal level of activity that is enhanced by specific binding of MVC(s) to a site(s) distal to the active site. For example, Na^+ binding to thrombin induces subtle conformational changes that propagate through the protein to the active site to promote catalysis [86]. A contrasting example is the DNA mismatch repair protein, MutL, in which a MVC is bound very close to its substrate binding site and forms a water-mediated hydrogen bond with the α-phosphate of ATP [87]. Like afCT, MutL also exhibits a broad MVC specificity; although it has a preference for Na^+, it is also activated by K^+, Rb^+ and Cs^+. Given that the MVC is not in direct contact with substrate, and requirement for MVCs is not absolute, afCT can be clearly classified as a type II activated enzyme.

We suggest that the MVC bound by afCT activates the enzyme in two ways: (1) by increasing affinity for substrate via a water-mediated hydrogen bond and stabilization of the adenosine binding loop, and (2) by stabilization of the loop containing the catalytic histidine (His354). Interestingly, the k_{cat}/K_m of afCT was markedly increased by addition of either Na^+ or K^+. Both decreased the K_m for AcAc-CoA equally, but k_{cat} was 3-fold higher for K^+ than for Na^+. Assuming the rate of catalysis is much slower than substrate binding, this indicates that both ions increase substrate affinity similarly, but K^+ is more effective at promoting catalysis. Further biochemical studies are required to confirm this. It is known that ACATs can turn over AcAc-pantetheine with only slightly lower catalytic efficiency than AcAc-CoA [21, 23], indicating that the adenosine part of CoA is not required for substrate

binding. Comparison of the kinetic properties of afCT using CoA substrate versus pantetheine substrate would be valuable for delineating relative contributions of effects (1) and (2) to enhancing its catalytic efficiency. In addition, determination of the structure of the enzyme in the absence of MVCs and in complex with Na^+, followed up mutagenesis studies, is anticipated to provide further insight into the allosteric activation of afCT (and T2) by MVCs.

To our knowledge, ACATs are a unique example of a class of enzymes that includes members that are specifically activated by MVCs and members that are ion-independent, whilst otherwise maintaining a highly conserved overall structure. From our structural comparisons, we suggest that the structural determinants that differentiate K^+-activated ACATs (afCT and hT2) from ion-independent ACATs (hCT and bCT) involve very few residues. These include Tyr187 (afCT), which is a glutamine in both hCT and bCT, and Glu224, which is an aromatic residue in hCT and bCT. A previously reported clinical mutation in hT2 indicates the importance of Tyr187 (hT2 Tyr219): Biochemical characterization of hT2(Tyr219His) showed that this mutation abolished enzyme activity [31]. Glu224 confers a strong negative charge to facilitate electrostatic attraction of K^+ to the MVC coordination site (Figure S7A). In addition to these two residues, the Lβ3-Lα3 loop, which forms the outside of the adenosine binding pocket, is one residue longer in these ion-independent ACATs. This additional histidine (hCT His224) causes a structural rearrangement allowing a cation-π interaction between the preceding basic residue (hCT Arg223) and the adenine rings of CoA (Figure S9B). It is interesting to note that this basic residue is conserved in K^+-activated ACATs (afCT Lys226), but the conformation of the Lβ3-Lα3 precludes it from contacting the substrate.

Insights into other species. Surprisingly, afCT is more like hT2 than hCT or bCT in terms of primary sequence, structure of key elements surrounding the active site, and biochemical properties. Discrimination of mitochondrial ACAT from cytosolic ACAT based on activation by K^+ – as demonstrated for mammalian tissues [29] – clearly does not apply for fungi. It is interesting to note that *A. thaliana* cytosolic ACAT is also more similar to hT2 and afCT than to hCT and bCT in terms of overall sequence identity (Figure S1) and conservation of key residues involved in substrate specificity and binding of K^+ and Cl^- (Figure S2). It is therefore possible that the biochemical properties of plant ACATs also conflict with the current paradigm differentiating mitochondrial and cytosolic ACATs, and that bacterial and mammalian cytosolic ACATs are the exception regarding non-activation by K^+. Further bioinformatic and biochemical studies are anticipated to elucidate the extent to which MVC-activation of ACATs is conserved across all domains of life. To our knowledge, there is no published data showing that the activity of T2 is independent of Na^+ ions. The similarity between the ion binding sites of hT2 and afCT along with the MVC promiscuity that afCT exhibits leads us to suggest that the accepted notion that T2 is activated by K^+ but not Na^+ [29] may need to be revisited. That is, although K^+ is the preferred activating MVC, afCT – and therefore probably T2 – are not strictly selective for K^+, and thus do not adhere to a strict dichotomy of activation by K^+ but not Na^+.

CONCLUDING REMARKS

We have solved the first structure of a fungal type II thiolase, both in apo form and in complex with substrate. The different catalytic site configurations trapped in the crystals of afCT provide the first direct experimental observations of the structures of the tetrahedral intermediates formed during the thiolase reaction cycle. We have also shown that, unexpectedly, afCT possesses specific ion binding sites and is activated by MVCs in a similar way to hT2. Future work focusing on the comparison of key structural features of ion-dependent and ion-independent thiolases is anticipated to provide further structural rationale for the allosteric ion-activation of this class of enzymes.

Biosynthetic thiolases catalyze the first step in the synthesis of highly reduced organic molecules in both prokaryotes and eukaryotes that are essential for energy storage and maintenance of normal cellular function, presenting them as potential drug targets. In addition, for many years much biotechnological research has focused on microbial metabolic engineering for the large-scale biosynthesis of biodegradable plastics such as polyhydroxyalkanoates [88-91], high value isoprenoid-based

natural products [16], and production of biofuels from renewable sources [92-95] – all processes for which biosynthetic thiolases play a central role. Thus, the detailed structural investigation of ACATs, such as presented herein, is anticipated to inform both the rational design of antimicrobial drugs, and the advancement of biotechnological applications for industrial, commercial and biomedical fields in the future.

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Author Contributions

Andrew Marshall. Purification of enzyme, enzymology, manuscript preparation, crystallography.

Charlie Bond. Crystallography, manuscript preparation, and intellectual contribution.

John Bruning. Crystallography, management of project, manuscript preparation, and intellectual contribution.

Notes

The authors declare no competing financial interest.

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ASSOCIATED CONTENT

Supporting Information

X-ray crystallographic data collection and structure refinement statistics (Table S1); map peak heights for anomalous data and F_o-F_o data, and atomic properties for all modeled Cs^+ and Rb^+ ions (Tables S2 and S3); atomic properties for all modeled K^+ and NH_4^+ ions (Table S4); supporting information figures S1-S11.

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FIGURES

Figure 1. Production of mevalonate in the cytosol, involving three steps starting with the condensation of two molecules of acetyl-CoA by acetoacetyl-CoA thiolase (ACAT). Mevalonate is the precursor for all isoprenoids, including ergosterol, for which the chemical structure is shown (bottom).

Figure 2. The catalytic cycle of ACAT. Residue numbers refer to *A. fumigatus* cytosolic ACAT (afCT). Cys92, His354, Cys384 and Gly386 of afCT correspond to Cys89, His348, Cys378 and Gly380 of *Z. ramigera* cytosolic ACAT (bCT), respectively (see text). Arrows indicate progress of the reaction in the biosynthetic direction. Oxyanion hole 1 (OAH1) is formed by the sidechain of His354 and a water molecule (referred to as Wat_{cat1} herein); OAH2 is formed by backbone N atoms of Cys92 and Gly386. Note that the thioester oxygen atom of the tetrahedral intermediate formed at stage 3 is drawn occupying OAH2. This is consistent with the structural evidence contained herein, and challenges the previous understanding of the reaction mechanism, where it was thought that this tetrahedral intermediate was stabilized by OAH1 [36].

Figure 3. Activation of afCT by monovalent cations (MVC). Addition of 100 mM chloride salts of ammonium or all common alkali-metal ions (100 mM) increased afCT activity relative to when no additional salt was added to the standard assay conditions (see Experimental Section; "low salt" = approx. 0.08 mM NaCl from protein storage buffer). Addition of 100 mM choline chloride resulted in no increase in enzyme activity, showing that activation cannot be attributed to an increase in the Cl^- concentration or an increase in the ionic strength. The ionic radius of each MVC (coordination number = 6) is shown (values are from the Database of Ionic Radii (http://abulafia.mt.ic.ac.uk/shannon/) [96]; NH_4^+ ionic radius is from Sidey (2016) [97]). Enzyme activity values are the mean of four separate experiments (except for NH_4Cl; $n = 3$). Error bars represent standard error of the mean.

Figure 4. Cartoon representation of the three-dimensional structure of afCT showing CoA (cyan sticks), K^+ ion (purple spheres) and Cl^- ion (green spheres) binding sites. **(A)** Tetrameric arrangement, with each subunit colored differently. **(B)** Subunit tertiary structure. α-helices (solid cylinders) and β-strands (arrows) are labeled according to Modis and Wierenga (1999) [24]. The NTD, loop domain and CTD are colored red, green and blue, respectively. Other subunits are colored white.

Figure 5. Stereo view of the monovalent cation binding site adjacent to the substrate binding pocket of afCT. The K^+ (purple sphere) is coordinated by adenosine pocket residues and Val350 (marked with asterisks), which is in the loop preceding the catalytic histidine (His354). Coordination bond (red dashes) lengths are shown (Å). afCT apo (light green) and liganded (dark green) are superposed, showing that the positions of the K^+ and surrounding residues are unaltered by ligand binding, but Wat_{MVC} (red sphere) shifts closer to CoA (cyan) in order to mediate hydrogen bonding from K^+ to CoA(N7A) (black dashes).

Figure 6. Stereo view of the Cl^- binding site at the dimerization interface of afCT with key residues labeled. Catalytic residues are labeled in bold type. Main chain atoms are hidden for all residues except Gly386 and Gly389. Opposing subunits of the tight dimer are colored different shades of green. The Cl^- ion (green sphere) is coordinated by three protein atoms and two waters (red spheres) near the thiolase

catalytic site. Lengths of coordination bonds (red dashes) and water-mediated hydrogen bonds (black dashes) to residues adjacent to the catalytic site are displayed in Å. Wat_{CL1}, Wat_{CL2} and Wat_{CL3} are numbered. An anomalous difference map calculated from data collected at 8500 eV is shown for the Cl^- as blue mesh contoured at 3σ. Residues 85-86 were excluded from the foreground for clarity.

Figure 7. Mode of CoA binding. **(A)** Stereo view of CoA substrate bound to afCT. Key residues are shown as sticks. A 2mFo-DFc composite omit map (blue mesh) contoured at 1.0 σ is shown for the CoA molecule. The K^+ is represented by a purple sphere. **(B)** Two-dimensional schematic of interactions between afCT and bound CoA. Hydrogen bonds are indicated by dashed lines, van der Waals contacts are indicated by solid curved lines. Waters involved in water-mediated hydrogen bonds are black circles. All interacting residues are from the same subunit, except for Tyr138 (marked with an asterisk), which protrudes for the tetramerization loop of the diagonally opposite subunit.

Figure 8. afCT catalytic site shown in stereo view. Superposition of afCT liganded (dark green) and apo (light green) structures shows that ligand binding induces changes in the sidechain conformations of Cys92 and Met161. Cys92 is acetylated in the liganded structure. Key residues are labeled and key waters represented by spheres; colored red in the liganded structure and light green in the apo structure. Positions of Wat_{cat1}, Wat_{cat2} and Wat_{cat3} are labeled '1', '2' and '3'. Wat_{cat1} shifts into OAH1 in the absence of an occupying acetyl group (i.e. from Ac-CoA); this position is labeled '0'. Asterisks indicate alternate conformations for waters modeled in the liganded structure (see text). OAHs 1 and 2 are indicated in the liganded structure by hydrogen bonds (red dashes; measurements are in Å) to Wat_{cat1} (position '0') and Ac-Cys92, respectively. When occupying position '1', Wat_{cat1} is stabilized by a hydrogen bond from Asn322 (black dashes).

Figure 9. Stages 2 and 4 of the thiolase reaction cycle trapped in afCT crystal structures. Images are shown in stereo. Polder maps (blue mesh) are shown for: Cys92 sidechain, the sulfur and adjacent carbon of CoA, acetyl groups, and waters in positions 0, 1 or 2 of the water trail. Maps are contoured at 3.0σ. Waters with alternate conformations are marked with asterisks. Hydrogen bonds of OAHs are indicated by red dashes. The hydrogen bond from Asn322 stabilizing Wat_{cat1} is colored black. Units of measurement are Å. **(A)** Catalytic site of afCT+AcCoA+K^+ subunit D demonstrates the conformation of Ac-Cys92 occupying OAH2 with CoA bound. Wat_{cat1} partially occupies OAH1. **(B)** Catalytic site of afCT+AcCoA+K^+ subunit C shows the position of the acetyl group both before and after the first acetyl-transfer reaction as alternate conformations of Ac-CoA (occupying OAH1) and Ac-Cys92 (occupying OAH2). His354 (foreground) is shown as transparent sticks.

Figure 10. Both tetrahedral reaction intermediates of the thiolase reaction cycle trapped in afCT crystal structures. Images are shown in stereo. Polder maps (blue mesh) are shown for: Cys92 sidechain, the sulfur and adjacent carbon of CoA, acetyl groups, and waters in positions 1 or 2 of the water trail. Maps are contoured at 3.0σ and 2.5σ for (A) and (B), respectively. The hydrogen bond from Asn322 stabilizing Wat_{cat1} is colored black. Units of measurement are Å. **(A)** Catalytic site of afCT+AcCoA+NH_4^+ subunit D shows the covalent tetrahedral intermediate formed during the first acetyl-transfer step, stabilized by OAH2. Dashed sticks indicate bonds that are formed or broken during this stage of the thiolase reaction cycle. **(B)** Catalytic site of afCT+AcCoA+NH_4^+ subunit C shows the covalent tetrahedral intermediate formed during the second acetyl-transfer step. The first acetyl group is stabilized by OAH2, and the second (Ac-CoA) occupies OAH1. Superposition with AcAc-CoA bound to bCT (Cys98Ala) (narrow salmon sticks; PDB: 1M1O) shows that the intermediate is positioned in a similar way to AcAc-CoA, as expected.

TABLES

Table 1. Kinetic Parameters of *A. fumigatus* cytosolic ACAT (afCT) (this study), human cytosolic ACAT (hCT) [74], rat liver cytosolic ACAT (rCT) [53], human mitochondrial ACAT (hT2) [30] and *Z. ramigera* cytosolic ACAT (bCT) [37] with acetoacetyl-CoA as the substrate.

Enzyme	Conditions[b]	K_m (μM)	k_{cat} (sec^{-1})	k_{cat}/K_m ($sec^{-1}.\mu M^{-1}$)
afCT[a]	low salt[c]	42 ± 19	7 ± 2	0.17
	100 mM NaCl	8 ± 2	33 ± 3	4.1
	100 mM KCl	8 ± 2	100 ± 10	13
hCT [74]	low salt	ND[d]	~ 170	ND
rCT [53]	50 mM KCl	33	ND	ND
hT2 [30]	40 mM NaCl	8 ± 2	7.4 ± 0.2	0.93
	40 mM KCl	4 ± 0.6	21 ± 1	5.3
bCT [37]	low salt	24 ± 5	813 ± 125	34

[a] All data for the current study were collected in triplicate, with uncertainty expressed as standard error. [b] All assays were done in the thiolytic direction using the Mg^{2+} method under similar conditions, but with various [salt]. [c] Salts included in standard condition for the current study are 20 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.1), and approx. 0.08 mM NaCl (from protein storage buffer). [d] Not determined.

Table 2. Average Rb and Cs anomalous peak heights (RMSD units, σ) for data collected at different wavelengths.

Element	8500 eV data	13000 eV data	15350 eV data
Rb	NA[a]	4.5	13.2
Cs	23.5	11.9	NA

[a] Not applicable.