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Insights into the stability and substrate specificity of the *E. coli* aerobic β -oxidation trifunctional enzyme complex

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Running Title

Structural and functional studies of the *E. coli* aerobic trifunctional enzyme (EcTFE)

Abstract

Degradation of fatty acids by the β -oxidation pathway results in the formation of acetyl-CoA which enters the TCA cycle for the production of ATP. In *E. coli* the last three steps of the β -oxidation are catalyzed by two heterotetrameric $\alpha_2\beta_2$ enzymes namely the aerobic trifunctional enzyme (EcTFE) and the anaerobic TFE (anEcTFE). The α subunit of TFE has 2*E*-enoyl-CoA hydratase (ECH) and 3*S*-hydroxyacyl-CoA dehydrogenase (HAD) activities whereas the β subunit is a thiolase with 3-ketoacyl-CoA thiolase (KAT) activity. Recently, it has been shown that the two TFEs have complementary substrate specificities allowing for the complete degradation of long chain fatty acyl-CoAs into acetyl-CoA under aerobic conditions. Also, it has been shown that the tetrameric EcTFE and anEcTFE assemblies are similar to the TFEs of *Pseudomonas fragi* and human, respectively. Here the properties of the EcTFE subunits are further characterized. Strikingly, it is observed that when expressed separately, EcTFE- α is a catalytically active monomer whereas EcTFE- β is inactive. However, when mixed together active EcTFE tetramer is reconstituted. The crystal structure of the EcTFE- α chain is also reported, complexed with ATP, bound in its HAD active site. Structural comparisons show that the EcTFE hydratase active site has a relatively small fatty acyl tail binding pocket when compared to other TFEs in good agreement with its preferred specificity for short chain 2*E*-enoyl-CoA substrates. Furthermore, it is observed that millimolar concentrations of ATP destabilize the EcTFE complex, and this may have implications for the ATP-mediated regulation of β -oxidation in *E. coli*.

Keywords

Fatty acid β -oxidation, trifunctional enzyme, TFE, ATP, substrate channeling, thiolase, MFE1

List of Abbreviations

AD: acyl-CoA dehydrogenase

anEcTFE: *E. coli* anaerobic TFE

ArcA: aerobic respiration control protein

BN-PAGE: blue native PAGE

CAA: acetoacetyl-CoA

CD: circular dichroism

CRP-cAMP: cAMP-activated global transcriptional regulator

CO8: octanoyl-CoA

DTT: dithiothreitol

E. coli: *Escherichia coli*

ECH: 2E-enoyl-CoA hydratase

EcTFE: *E. coli* aerobic TFE

FadR: fatty acid metabolism regulator protein

HAD: 3S-hydroxyacyl-CoA dehydrogenase

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HSC: 3S-hydroxydecanoyl-CoA

HsTFE: human mitochondrial TFE

KAT: 3-ketoacyl-CoA thiolase

MFE1: multifunctional enzyme, type-1

MtTFE: *Mycobacterium tuberculosis* TFE

OmpR: DNA- binding dual transcriptional regulator

PfTFE: *Pseudomonas fragi* TFE

RnECH: *Rattus norvegicus* ECH

RnMFE1: *Rattus norvegicus* MFE1

SAXS: small angle X-ray scattering

SEC: size exclusion chromatography

SLS: static light scattering

StTFE: *Salmonella typhimurium* TFE

TFE: trifunctional enzyme

T_m : melting temperature

V_M : Matthew's coefficient

Introduction

The Coenzyme A (CoA) conjugated fatty acids are degraded via the fatty acid β -oxidation pathway in which acetyl-CoA is released in each cycle until the chain length of the fatty acyl-CoA is no longer than two or three carbons. When linear fatty acyl-CoAs are oxidized, the end product is acetyl-CoA, which is a substrate of subsequent metabolic pathways such as TCA cycle for ATP production or biosynthetic pathways. The methyl branched fatty acyl-CoAs are broken down to finally yield propionyl-CoA, which is further metabolized to succinyl-CoA that enters the TCA cycle. *E. coli* has two parallel fatty acid β -oxidation pathways, one is referred as the aerobic β -oxidation pathway where oxygen acts as the electron acceptor and the other as the anaerobic β -oxidation pathway, in which nitrate or fumarate are the electron acceptors (Campbell et al., 2003, Snell et al., 2002). Under aerobic conditions both pathways are active, while under anaerobic conditions only the anaerobic pathway is functional. β -oxidation is a highly regulated pathway under the control of various transcriptional factors in *E. coli* (Fujita et al., 2007). For example, fatty acid metabolism regulator protein (FadR) allows the expression of β -oxidation enzymes only in the presence of long chain fatty acids (DiRusso and Nunn 1985, DiRusso et al., 1992, Black and DiRusso 1994). Aerobic respiration control protein (ArcA) represses the expression of aerobic β -oxidation enzymes under anaerobic conditions (Cho et al., 2006, Feng and Cronan 2010). DNA-binding dual transcriptional regulator (OmpR) regulates β -oxidation by controlling the expression of enzymes required for the uptake of long chain fatty acids (Higashitani et al., 1993). cAMP-activated global transcriptional regulator (CRP-cAMP) activates the expression of β -oxidation enzymes in the absence of glucose (Feng and Cronan 2012, Fic et al., 2009) to make use of fatty acids as a source of carbon and energy.

In *E. coli*, the first reaction of the four steps of fatty acid β -oxidation is catalyzed by acyl-CoA dehydrogenase (AD) encoded by the genes *fadE* and/or *ydiO*. The next three reactions are catalyzed by two trifunctional enzyme (TFE) complexes namely EcTFE (encoded by *fadBA*) and anEcTFE (encoded by *fadIJ*). Both complexes are $\alpha_2\beta_2$ -heterotetramers, in which the α -subunits, have 2E-enoyl-CoA hydratase (ECH) and 3S-hydroxyacyl-CoA dehydrogenase (HAD) active sites and the β -subunits, have 3-ketoacyl-CoA thiolase (KAT) activity (Binstock and Schulz 1981) (Figure 1). It has been proposed, based on phylogenetic sequence analysis, that there exist at least four different TFE-subfamilies, having different assemblies (Venkatesan and Wierenga 2013). High resolution structures of *Pseudomonas fragi* TFE (PfTFE) (Imamura et al., 1990, Ishikawa et al., 2004), *Mycobacterium tuberculosis* TFE (MtTFE) (Venkatesan and Wierenga 2013) and human mitochondrial TFE (HsTFE) (Liang et al., 2018, Xia et al., 2019) show that these three TFEs indeed have unique quaternary structures. Interestingly, in *P. fragi*, *M. tuberculosis* and human, there is only one TFE whereas in *E. coli* there are two TFEs (Campbell et al., 2003, Sah-Teli et al., 2019). Recently, the low resolution SAXS and EM structures of these two TFEs have been reported (Sah-Teli et al., 2019), showing that the overall tetrameric assemblies of EcTFE and anEcTFE are similar to the PfTFE and HsTFE assemblies, respectively. The kinetic properties of anEcTFE and HsTFE are also similar in that they prefer longer chain fatty acyl-CoAs whereas EcTFE prefers shorter to medium chain length fatty acyl-CoAs. Such complementary substrate specificities of EcTFE and anEcTFE allow for a complete degradation of fatty acyl-CoAs in *E. coli* under aerobic conditions (Sah-Teli et al., 2019) while in human mitochondria the shorter chain fatty acyl-CoAs are metabolized by monofunctional β -oxidation enzymes (Houten et al., 2016).

In order to understand these different substrate specificities better, here we report on properties of the α - and β -subunits of EcTFE, including the crystal structure of the EcTFE- α subunit. It is found that while both chains are less stable when purified individually, EcTFE- α is active whereas EcTFE- β is completely inactive in the absence of EcTFE- α . The structural analysis shows that EcTFE- α has a relatively small fatty acyl binding pocket in its hydratase active site mainly due to the presence of a bulky amino acid (Trp87) lining its binding pocket. In these *in vitro* studies, it is also observed that in the presence of ATP the EcTFE complex is destabilized and EcTFE- β is inactivated. This property can possibly have relevance for the regulation of β -oxidation *in vivo* in *E. coli*.

Material and Methods

Cloning, overexpression and purification

The genes *fadB* (Gene Accession Number: ECK3838) encoding EcTFE- α and *fadA* (Gene Accession Number: ECK3837) encoding EcTFE- β were amplified by PCR from the genomic DNA of *E. coli* K12 MG1655 strain as the template with appropriate primers (Supplementary Table 1). *fadB* was inserted in the multiple cloning site (MCS) 1 after 6xHis-tag coding sequence in pETDuet-1 expression vector (EcTFE- α -His construct) and *fadA* was inserted in the MCS2 of the pACYC-Duet-1 expression vector (EcTFE- β construct). Similarly, *fadA* was inserted in the MCS1 after 6xHis-tag coding sequence in pETDuet-1 expression vector (EcTFE- β -His construct) using the restriction free PCR approach. The α W87G-EcTFE- α -His variant was generated by site-directed mutagenesis using appropriate primers (Supplementary Table 1). EcTFE- α -His and EcTFE- β were coexpressed in *E. coli* BL21DE3 to obtain the EcTFE complex. Similarly, α W87G-EcTFE- α -His and EcTFE- β were coexpressed to obtain the α W87G-EcTFE variant of the complex. EcTFE- α -His and EcTFE- β -His were overexpressed in *E. coli* BL21DE3pLys. The overexpressed individual subunits as well as the complexes were purified as reported previously (Sah-Teli et al., 2019). Briefly, the soluble fraction containing the overexpressed protein was incubated with Ni-NTA beads, washed and the protein was then eluted with a buffer containing 50 mM Tris, 0.5 M NaCl, 10% glycerol, and 250 mM imidazole at pH 8.0. The eluted protein was concentrated and passed through Superdex 200 Hiload 16/60 column (GE Healthcare) pre-equilibrated with 20 mM HEPES buffer with 120 mM KCl, 2.5 mM DTT at pH 7.2. The peak fractions were pooled, concentrated and stored at -70 °C for further use. Similarly, MtTFE was overexpressed in *E. coli* BL21DE3 and purified by Ni-NTA affinity chromatography followed by size exclusion chromatography (SEC) as described previously (Venkatesan and Wierenga 2013).

Static Light Scattering (SLS)

The protein solution (50 μ L, 5 mg mL⁻¹) was injected onto a Superdex 200 Increase 10/300 GL column (GE Healthcare), connected to a Shimadzu purification system, a Shimadzu refractive index (RI) detector and a Wyatt mini-DAWN Treos SLS detector. The Astra software was used to calculate the molecular mass of the protein of the eluted peak.

Circular Dichroism (CD) spectroscopy

Purified protein (0.1 mg mL⁻¹) in 10 mM potassium phosphate buffer pH 7.6 was used for CD measurements using the Chirascan CD spectrophotometer (Applied Photophysics). The secondary

structure content was calculated from the far UV CD measurements using the CDNN software. The melting temperature (T_m) of the sample was determined using the Global3 software by comparing the spectra from 195 to 260 nm obtained by heating the sample at a rate of either 1 or 2 °C per min from 22 to 94 °C. In addition, the EcTFE and EcTFE- β were preincubated with varying concentrations of ATP or ADP on ice for 30 min and the CD measurements of these samples were recorded in a similar way.

Blue Native PAGE (BN-PAGE)

The mini-PROTEAN TGX precast gradient (4-15%) gels (BIORAD) were used for BN-PAGE. Approximately 50 μ g of protein solutions preincubated with various concentrations of ATP or ADP or NAD^+ , were mixed with sample buffer containing 0.1% Coomassie G-250 without SDS and β -mercaptoethanol and loaded in the sample wells. The samples were run for 2-3 hours at 80 volts (Wittig et al., 2006).

Reconstitution of the heterotetrameric EcTFE complex from its individual subunits

Separately expressed and purified EcTFE- α and EcTFE- β in 20 mM HEPES buffer with 120 mM KCl, 2.5 mM DTT at pH 7.2, were mixed in 1:1 molar ratio and incubated on ice for 30 min. The resulting mixture was analyzed by SEC and by enzymatic assays.

Enzyme activity measurements

The enzyme activity measurements of EcTFE, EcTFE- α , EcTFE- β and α W87G-EcTFE were performed at 25 °C using the same protocols and assay buffers as described previously (Sah-Teli et al., 2019) using the Jasco V660 spectrophotometer. The effects of ATP or ADP or NAD^+ , were studied by preincubation of the enzyme solution in the gel filtration buffer with these compounds for 30 minutes on ice before diluting the enzyme into the enzyme assay mixture. The ECH and HAD activities of EcTFE and EcTFE- α samples, preincubated with different concentrations of ATP or ADP or NAD^+ were measured at 263 and 340 nm, respectively, using 60 μ M 2*E*-butenoyl-CoA as substrate in the assay buffer (50 mM Tris, 50 mM KCl, 50 μ g mL⁻¹ BSA, pH 9.0). The HAD reaction mixture also contained 1 mM NAD^+ and 1 mM CoA. The KAT activity of EcTFE- β , reconstituted EcTFE complex from its subunits and EcTFE, preincubated with varying concentrations of ATP or ADP or NAD^+ was measured at 303 nm using 60 μ M acetoacetyl-CoA as substrate in the assay buffer (50 mM Tris, 50 mM KCl, 50 μ g mL⁻¹ BSA, pH 8.5) containing also 1 mM NAD^+ , 0.5 mM CoA and 5 mM MgCl₂. The molar extinction coefficients of 6700 M⁻¹ cm⁻¹, 6200 M⁻¹ cm⁻¹, 21400 M⁻¹ cm⁻¹ of 2*E*-butenoyl-CoA, NADH and the Mg²⁺-acetoacetyl-CoA complex, respectively, were used to calculate the specific activities for the ECH, HAD and KAT reactions, respectively. Similarly, the specific activity measurements were carried out for α W87G-EcTFE with 2*E*-butenoyl-CoA, 2*E*-decenoyl-CoA and 2*E*-hexadecenoyl-CoA for ECH and HAD activities and with acetoacetyl-CoA for KAT activity, using 60 μ M concentrations. All these measurements have been done at least twice. The specific activities were then compared with that of the wild type EcTFE complex.

Crystallization of EcTFE and EcTFE- α

EcTFE (or EcTFE- α ; 5 mg mL⁻¹) was crystallized in TTP plate using the sitting drop vapor diffusion method using 100 mM Bis-Tris propane, pH 7.5, 25% w/v PEG 3550, 367 mM KNO₃ and 25 mM ATP at 4 °C (Table 1). The formation of crystals was monitored using the in-house IceBear software.

The crystals appeared after one month and were allowed to grow further for 2-6 months before they were harvested for diffraction studies. For cryo protection, the crystals were moved quickly through the cryo protection buffer (reservoir solution supplemented with 20% v/v glycerol) and subsequently frozen in liquid nitrogen (Table 1).

EcTFE- α crystal structure determination

The X-ray diffraction data were collected at beam lines P14 at PETRA III, EMBL Hamburg and at I04 at Diamond Light Source, UK. The crystals diffracted to a maximum resolution of 2.95 Å. The acquired data set was reduced and scaled by the xia2-DIALS package (Winter et al., 2018), and was merged and analyzed by AIMLESS (Evans and Murshudov 2013) of the CCP4i package. The structure was solved by molecular replacement (in MoRDa, CCP4 Cloud) (Vagin and Lebedev 2015) in the C222₁ space group using PfTFE- α (PDB code: 1WDK, chain A) subunit as the template. Iterative cycles of structure refinement and manual model building were carried out using phenix.refine of the PHENIX suite (Liebschner et al., 2019) and COOT (Emsley et al., 2010) respectively. The data processing and structure refinement statistics are reported in Table 1, and the coordinates have been deposited in the Protein Data Bank with the accession code 6TNM.

Structure analysis, modelling and sequence alignment

The structures were analysed with COOT and other CCP4 programs (Cowtan et al., 2011, Winn et al., 2011). The figures were made with PyMOL (the PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). Superpositions were done with COOT using the SSM option (Krissinel and Henrick 2004). The sequence alignments were made with Clustal Omega (Thompson et al., 1994) and ESPript 3.0 server (Robert and Gouet 2014). The EcTFE tetramer was obtained by using the PfTFE structure (PDB code:1WDK) as a reference model for superimposing the structures of the EcTFE- α chain and the EcTFE- β dimer modeled by SWISSMODELLER using the 1.7 Å resolution crystal structure of the *Salmonella typhimurium* TFE- β (StTFE- β) dimer (PDB code: 3GOA) as the template. The sequence of the latter thiolase is 95% identical to the EcTFE- β . Therefore in the model of the tetramer both chains have been completely built, including their side chains. The electrostatic surfaces were calculated from this model with CCP4MG (McNicholas et al., 2011). The shape and solvent accessible volume of the acyl binding pockets of the ECH active sites were calculated using PyMOL and CASTp online tool (Tian et al., 2018) respectively. The shape properties of the ECH acyl binding pocket of EcTFE- α were studied by comparison with several homologous structures, in particular PfTFE, MtTFE and HsTFE. In the latter structures the mode of binding of the acyl tail in the respective binding pockets was obtained by superimposing the structure of the RnMFE1 complexed with 3S-hydroxydecanoyl-CoA (HSC) (PDB code: 3ZWC, chain A) on the ECH active site. The crystal structures of the RnECH complexes with bound acetoacetyl-CoA (CAA) (PDB code: 1DUB, chain A) and octanoyl-CoA (CO8) (PDB code: 2DUB, chain A) were also used for these comparisons. The NAD⁺ binding pocket of the EcTFE HAD active site was identified by superimposing the RnMFE1 structure (PDB code: 3ZWC, chain A) complexed with NAD⁺ on the HAD active site.

Results

EcTFE- α exists as a monomer in solution

EcTFE- α was expressed with N-terminal 6xHis-tag and was purified using affinity chromatography and SEC. The SEC profile of EcTFE- α showed three peaks at 45, 62 and 79 mL in Superdex 200 Hiload 16/60, 120 mL column (Figure 2a). The peaks at 45 and 62 mL correspond to soluble aggregates and higher oligomers of EcTFE- α respectively. The fractions from major peak around 79 mL were pooled, concentrated and used for further studies. CD measurements of EcTFE- α show that the protein is folded and consists of 37% α -helix, 31% β -strand and 32% random coil with a T_m of 43 °C (Figure 2b-c). The molecular mass of EcTFE- α is about 71 kDa as calculated from the SLS experiment (Supplementary Figure 3a), which is close to its theoretical monomeric molecular mass (78 kDa). It has a predominant band between 66 and 133 kDa in BN-PAGE (Figure 3a).

EcTFE- β is a mixture of monomers and dimers

The EcTFE- β was also expressed with N-terminal 6xHis-tag and purified similarly as EcTFE- α . It elutes at 82 mL in Superdex 200 Hiload 16/60, 120 mL column (Figure 2a), corresponding to a molecular mass significantly lower than EcTFE- α , which elutes at 79 mL. It is folded and consists of 37% α -helix, 32% β -strand and 31% random coil as exhibited by CD measurements (Figure 2b). The T_m of EcTFE- β was calculated to be 45 °C from the CD melting curves (Figure 2c). The molecular mass calculated for EcTFE- β by SLS is about 75 kDa (Supplementary Figure 3b), while the theoretical monomeric molecular mass of EcTFE- β is 42 kDa. A diffuse band of EcTFE- β is observed, approximately from 133 kDa to below 66 kDa, in BN-PAGE (Figure 3b). These results suggest that EcTFE- β exists as a mixture of dimers and monomers in the purified condition.

EcTFE- β is functional only in the EcTFE complex

As shown in the above section, EcTFE- α and EcTFE- β can be purified. However, both exhibit lower T_m (Figure 2c) than the EcTFE complex (T_m = 43 °C and 45 °C, respectively, instead of 51 °C), showing that the heterotetrameric complex is the most stable form. In addition, the ECH and HAD specific activities of EcTFE- α with 2*E*-butenoyl-CoA are 13.8 ± 2.5 and 1.8 ± 0.3 $\mu\text{mole mg}^{-1} \text{min}^{-1}$ respectively, whereas the corresponding activities in the complex are 29 ± 2 and 2.7 ± 0.4 $\mu\text{mole mg}^{-1} \text{min}^{-1}$ (Sah-Teli et al., 2019). That is, EcTFE- α is less active than when present as part of the EcTFE complex (Figure 2e). Moreover, surprisingly, EcTFE- β (with or without 6xHis-tag) did not have any KAT activity when purified alone (Figure 2e), although it could be purified as a soluble, folded protein. Since the enzyme activities of EcTFE- α as well as EcTFE- β are affected when purified alone, the purified subunits were mixed and incubated together *in vitro*. Interestingly, the KAT specific activity was regained to about 50% of the EcTFE complex KAT activity (Figure 2e). Further, the mixed subunits when passed through SEC column elute as a peak corresponding to the EcTFE complex (Figure 2d). These results clearly demonstrate that the EcTFE complex can be reconstituted *in vitro* and the complex is more active than the separate subunits. In particular, the improvement in the KAT activity in the reconstituted complex is striking. This can also be correlated with the SLS and BN-PAGE studies which show that EcTFE- β is probably a mixture of monomers and dimers. The dimer is the active form of thiolase (Kiema et al., 2019), which is stabilized in the EcTFE complex.

Selective crystallization of EcTFE- α from EcTFE solutions

Previously, we have determined the low resolution structure of EcTFE using SAXS and negative staining EM analysis (Sah-Teli et al., 2019). To determine the high-resolution structure of EcTFE,

crystallization experiments were carried out with EcTFE. After extensive screening and optimization, crystals of reasonable size (50 μm) were obtained. However, the crystals did not show any diffraction. In further optimization with various additive screens diffraction quality crystals of EcTFE were obtained in the presence of 25 mM ATP. Analysis of the crystal contents suggested a Matthews coefficient (V_M) of 2.2 $\text{\AA}^3/\text{Da}$ (44% solvent content) when considering one α and one β subunit in the asymmetric unit. However, attempts to determine the structure of EcTFE using PftTFE (~60% sequence identity for both subunits) did not give any feasible solutions. In most of the solutions suggested by PHASER, there were severe clashes between the α and β subunits. Mostly, the structure solution failed while trying to place the β subunit of EcTFE. This led us to suspect whether only one of the subunits of EcTFE (either a single molecule of EcTFE- α or a dimer of EcTFE- β) had been crystallized. Further molecular replacement calculations showed that the asymmetric unit consists of one EcTFE- α subunit ($V_M = 3.4 \text{ \AA}^3/\text{Da}$ and 63% solvent content). PISA analysis (Evgeny. 2010, Krissinel and Henrick 2007) of the EcTFE- α structure shows that the crystal packing does not generate dimers. The structure was subsequently refined to an R-factor and R-free of 0.2300 and 0.2711, respectively at 2.95 $\text{\AA}$ resolution. In effect, while trying to crystallize the EcTFE complex, only the EcTFE- α subunit had been crystallized.

Subsequently, experiments were carried out to understand why only the EcTFE- α subunit crystallized. The 6xHis-tag was on the EcTFE- α subunit in the purified EcTFE complex. This can lead to the presence of excess of uncomplexed EcTFE- α subunit. However, it should be noted that in SEC, the EcTFE- α subunit and EcTFE complex elute at different volumes, therefore, the complex could be separated from the uncomplexed EcTFE- α subunit. Noteworthy, only the crystals obtained in the presence of ATP diffracted well. Therefore, we reasoned that ATP could have some effect on the EcTFE complex. Also, in the refined structure the mode of binding of ATP was clearly defined by the electron density map being bound close to the NAD^+ binding site of the HAD part of EcTFE- α (Figure 6). These observations intrigued us to further investigate the mechanism and effect of ATP binding to EcTFE. In parallel, the effect of ADP and NAD^+ on the stability of the EcTFE complex was also tested.

Effect of ATP on the stability and activity of EcTFE

It was observed that when EcTFE was preincubated with ATP, the band corresponding to the EcTFE complex completely disappeared in BN-PAGE (Figure 3a). The two observed bands were only of EcTFE- α as confirmed by mass spectrometry. Further, when the EcTFE- β and EcTFE- α were preincubated separately with 5 mM ATP and ran on BN-PAGE, the band of EcTFE- β is not seen, whereas there is no effect on the EcTFE- α band (Figure 3b and 3d). These results suggest that ATP leads to the dissociation of the complex and precipitation of EcTFE- β . This experiment was repeated with MtTFE in which case the addition of 5 mM ATP had no effect (Figure 3a). Clearly, the ATP-induced dissociation of the complex is specific for EcTFE.

EcTFE preincubated with 5 mM ATP and EcTFE- β preincubated with 20 μM and 5 mM ATP were analyzed by CD experiments to understand the changes in their secondary structures and stability caused by ATP binding to the protein. EcTFE- β and EcTFE- β preincubated with 20 μM ATP has similar secondary structure features whereas these features are almost completely lost upon preincubation with 5 mM ATP (Figure 3e). The T_m of EcTFE- β and EcTFE- β preincubated with 20 μM ATP are 45 $^\circ\text{C}$ and 48 $^\circ\text{C}$, respectively whereas the T_m of EcTFE- β preincubated with 5 mM ATP

could not be measured as the secondary structure was lost in this sample. In case of the EcTFE complex preincubated with 5 mM ATP, the secondary structure features are similar when compared with EcTFE. However, the T_m of the former sample is reduced from 51 °C to 45 °C which is closer to the T_m of EcTFE- α showing that probably only EcTFE- β is precipitated from the EcTFE complex during the preincubation with 5 mM ATP, and EcTFE- α remains folded in solution even after dissociation. Moreover, the CD signal of EcTFE- β is lost only while preincubating with millimolar concentrations of ATP (5 mM), but no significant changes were observed with micromolar concentrations of ATP (20 μ M) (Figure 3e).

Similarly, enzyme activity measurements were carried out with EcTFE as well as EcTFE- α preincubated with ATP to understand better the effect of ATP on the enzyme activity. Preincubation of EcTFE with 5 mM ATP lead to 10 fold increase in the ECH specific activity and 3 fold increase in the HAD specific activity when 2*E*-butenoyl-CoA was used as substrate, whereas, for the EcTFE- α , there is no significant change (Figure 4a-b). It is possible that there is an allosteric communication between the two active sites of EcTFE- α such that ATP, upon binding to the HAD part, stabilizes the ECH part in a catalytically more competent form leading to the increased ECH activity. The higher ECH activity will increase the amount of available 3S-hydroxybutanoyl-CoA, thus also increasing the HAD activity. However, it is unclear why the ATP-induced increased ECH activity is not observed for the individually purified EcTFE- α . In any case, EcTFE- α is already less active when purified individually indicating that there are also other changes that are probably overriding the effect of interaction of ATP. Since addition of 5 mM ATP precipitated the EcTFE- β subunit from the complex, the KAT activity of EcTFE was also measured by preincubating the enzyme with varying concentrations of ATP ranging from 0.01-5 mM ATP. Up to 0.1 mM (100 μ M) ATP, the KAT activity was not affected much. However, preincubation with millimolar concentrations of ATP reduced the KAT activity by up to 90% (Figure 4c).

Effect of ADP and NAD⁺ on EcTFE

In parallel, the effect of ADP on the EcTFE, EcTFE- α and EcTFE- β was also studied by BN-PAGE, CD and enzyme assays. The BN-PAGE and CD experiments suggest that EcTFE- β does not dissociate from EcTFE upon preincubation with 5 mM ADP (Figure 3b-d). Also, ECH and HAD activities are not affected upon preincubation with 5 mM ADP (Figure 4a-b). However, preincubation with 5 mM ADP reduces the KAT activity of EcTFE in a similar manner as observed for EcTFE preincubated with ATP (Figure 4c). These results suggest that although ADP does not precipitate EcTFE- β , it affects the KAT activity of β -subunit of EcTFE like ATP does. Similarly, when NAD⁺ was preincubated with EcTFE and EcTFE- α , the ECH, HAD as well as KAT activities increased slightly (Figure 4a-b). This can be rationalised as NAD⁺ is the co-factor for the HAD subunit and binding of NAD⁺ may induce conformational changes in EcTFE that stabilize, respectively, the whole complex or EcTFE- α , resulting in overall higher activity whereas ATP and ADP negatively affect the KAT activity.

Crystal structure of EcTFE- α

The data collection, data processing and refinement statistics of EcTFE- α are shown in Table 1. There is one molecule in the asymmetric unit. The N-terminal 6xHis-tag and the C-terminal residues (10 residues) are disordered. The final model at 2.95 Å resolution contains residues from Met1 to Pro719.

The crystal structure of EcTFE- α shows the typical fold and domain structure as known from its homologues (Figure 5), including PfTFE, MtTFE, HsTFE and RnMFE1. The sequences of these chains are shown in Supplementary Figure 1. Five domains have been identified in this fold. Domains A and B make the ECH part (crotonase fold) and domains C, D and E make the HAD part. An interesting property of this fold concerns the notion that domain B is functionally an integral part of the crotonase fold, but structurally it is tightly associated with the D/E-domain of the HAD part. The analysis of the RnMFE1 structures has shown that two hinge regions can be identified (Kasaragod et al., 2013), being (i) between domain A and the BCDE-part, and (ii) between the C-domain and the D/E-domains. For both hinge regions “open” and “closed” states can be assigned using the distances between C_{α} -atoms of selected residues (Figure 5b), as is summarized in Table 2. In this EcTFE- α structure domain A as well as domain C are neither very closed, nor very open, like also observed for the α -chains of the PfTFE complex (Table 2).

The sequence comparison of EcTFE- α and RnMFE1 shows that the catalytic residues of the ECH part (Kasaragod et al., 2017) are fully conserved (Supplementary Figure 1). In the alignment of sequences of the crotonase fold enzymes there is much sequence length variability in the loop-2 region. For this region, which lines the binding pocket of the acyl moiety, the EcTFE loop-2 is more extended than in RnMFE1. In the HAD-part the catalytic residues (Kasaragod et al., 2017) are also fully conserved but the specificity loop of the HAD active site is shorter in EcTFE, as compared to RnMFE1 (Supplementary Figure 1). In RnMFE1 the two active sites are separated by 40 Å by a solvent exposed tunnel. This tunnel is shaped by the D/E-domains, as well as by the CH2A-CH2B helices, protruding out of the C-domain, and interacting directly with domain A. In EcTFE- α , the CH2A-CH2B helices are shorter and the tunnel like feature of RnMFE1 is absent.

In PfTFE, the two α -chains dimerise (being related by the local two fold axis of the tetramer) in such a way that the CH2A-CH2B helices interact with each other. When comparing the structures of EcTFE- α and PfTFE- α there are no structural differences in this region of the structure. The rms (C_{α}) difference for the region 344-379 (EcTFE- α) with region 345-380 (PfTFE- α , PDB code: 1WDK) for C_{α} -atoms is 1.1 Å using the LSQ option in COOT. The loop between CH2A and CH2B is not very well defined in the map, otherwise the superposition is very good. Also the two active site architectures are nearly the same, except for the structure of the region just before loop-2 of the ECH active site, which adopts a slightly different trace. This region has the same sequence in the PfTFE and EcTFE α -chains (Supplementary Figure 1). From homologous structures it is known that this region interacts with the adenine part of the CoA moiety and it also provides one of the two hydrogen bond donors of the oxyanion hole of the ECH active site (Zhang et al., 2010). In the PfTFE active site a detergent molecule is bound in this region, whereas in EcTFE- α a nitrate ion is bound, possibly mimicking competent active site hydrogen bond interactions with the oxyanion hole hydrogen bond donors as seen in other crotonase fold enzyme complexes. The conformation of this loop in EcTFE- α is the same as seen in other crotonase fold enzymes.

Mode of ATP binding

ATP is bound to domain C of the HAD part of the EcTFE- α . The topology of the C-domain conforms to the Rossmann fold extended by two additional β -strands (CB7 and CB8) (Supplementary Figure

1). The β -strands CB7 and CB8 run antiparallel to the parallel β -sheet of the Rossmann fold, formed by β -strands CB1-CB6. The N-terminal CB1-CH1-CB2 fold forms the ADP binding $\beta\alpha\beta$ -unit of the Rossmann fold and its sequence conforms to the sequence fingerprint of this unit, as found in NAD^+ binding enzymes (Wierenga et al., 1986). The mode of binding of NAD^+ to the HAD part, as observed for example in the structure of RnMFE1 complexed with NAD^+ is indeed the same as predicted from the sequence fingerprint properties (Kasaragod et al., 2013, Kasaragod et al., 2017).

The mode of binding of ATP is well defined by its electron density map (Figure 6). However, its mode of binding to the $\beta\alpha\beta$ -unit of the domain C of EcTFE- α is different from the mode of binding of NAD^+ . In the latter case the pyrophosphate of NAD^+ is tightly hydrogen bonded to the N-terminal region of the helix, whereas in this ATP mode of binding the phosphate moieties point away from the $\beta\alpha\beta$ -unit and its terminal phosphate group interacts with the opposing D/E-domains, being hydrogen bonded to the side chain atom NZ (Lys584) located between helix DH5 and the β -meander of the D-domain (Figure 6). This lysine is conserved in PfTFE and HsTFE, being Lys584 and Lys631, respectively (Supplementary Figure 1). Also, the mode of binding of the adenine moiety of ATP is different as compared to the NAD^+ mode of binding but the interactions of the ribose moiety being hydrogen bonded to Asp343 of the $\beta\alpha\beta$ -unit (Figure 6) are conserved.

The EcTFE- α binding pockets for the substrate fatty acyl moiety

EcTFE- α has two binding pockets for the acyl moiety of CoA, respectively, in its ECH and HAD parts. In the ECH domain, the binding pocket of the enoyl tail is lined by loop-2/helix-2. This region is very variable with respect to its sequence length (Supplementary Figure 1) (Onwukwe et al., 2015). The crystal structure of RnMFE1 complexed with 3S-hydroxydecanoyl-CoA (HSC) in its ECH active site was previously reported (Kasaragod et al., 2013). This structure was used as reference to analyze the shape of the ECH acyl binding pocket in the α -subunit of the TFEs. A careful comparison of the shape of the ECH active site binding pocket for the substrate enoyl chain shows that in EcTFE this pocket is narrower than in PfTFE, HsTFE, MtTFE and RnMFE1, due to the Trp87 side chain residue of Helix-2 (Figure 7). These acyl binding pockets are bulk solvent accessible in other TFEs whereas in EcTFE it is closed, mainly by the presence of the Trp87 side chain. It seems that this side chain would clash with the fatty acyl chain of longer substrates such as C_{12} or longer (Figure 7a). EcTFE has a substrate preference for short chain enoyl tails, but substrates with longer enoyl tails are also converted, but with approximately 100 fold lower rates (Sah-Teli et al., 2019). In order to investigate the importance of the Trp87 side chain for substrate specificity this residue was mutated into a glycine (as present in PfTFE at the same position). The α W87G-EcTFE complex has been purified and its properties were investigated. The α W87G-EcTFE variant is folded and properly assembled as suggested by SEC and CD secondary structure analysis but it is significantly less stable ($T_m = 45^\circ\text{C}$) than wild type EcTFE ($T_m = 51^\circ\text{C}$) (Supplementary Figure 6a-c). Both ECH and HAD activity of the α W87G-EcTFE variant with the substrates 2E-butenoyl-CoA, 2E-decenoyl-CoA, and 2E-hexadecenoyl-CoA are reduced by two to five fold in comparison to that of wild type EcTFE (Supplementary Figure 6d-e). Although the mutation is in the α -subunit of EcTFE, the α W87G-EcTFE variant has also exhibited lower KAT activity than that of wild type EcTFE with acetoacetyl-CoA as substrate (Supplementary Figure 6f). These experiments show that the W87G mutation makes the variant less stable and less active with substrates having different chain length. Apparently Trp87 alone is not the critical determinant of substrate specificity of EcTFE. In this respect it is interesting

to note the structural information from the crystal structures of the homologous rat mitochondrial monofunctional enzyme (RnECH) complexed with acetoacetyl-CoA (CAA, PDB code: 1DUB) and with octanoyl-CoA (CO8, PDB code: 2DUB), which have been previously reported (Engel et al., 1996, Engel et al., 1998) and the acyl binding pocket of RnECH was also analyzed (Figure 7f and Figure 8). In the acetoacetyl-CoA complex the pocket is small and not connected to bulk solvent (Figure 7f), but the residues Gly114, Lys115, Phe116, Leu117 and Ser118 of the loop-2/helix-2 region of RnECH are disordered in the RnECH structure, when complexed with octanoyl-CoA (Figure 8b), allowing for the binding of the longer acyl tail of octanoyl-CoA in this pocket. Possibly in EcTFE- α a similar conformational change as in RnECH in this region is triggered on binding of longer enoyl moieties, allowing EcTFE- α to use not only short chain but also medium and long chain 2E-enoyl-CoA as substrates, as is required by its enzyme kinetic properties. The acyl binding pocket in the HAD active site of EcTFE is more solvent exposed than its hydratase binding pocket. Structural comparisons show that the shape of this pocket is similar in all the analyzed TFEs and in RnMFE1.

Discussion

Expression and purification of the α -chain and β -chain separately, showed that both proteins can be purified individually. However, they are less stable compared to the EcTFE complex. The α -chain is purified as a catalytically competent monomer, whereas EcTFE- β exists as a mixture of monomers and dimers. Interestingly, the crystal structure of TFE- β from *Salmonella typhimurium* is available (PDB code: 3GOA). StTFE- β shares a sequence identity of 95% with the EcTFE- β and has been crystallized as a dimer. There are 19 sequence differences between EcTFE- β and StTFE- β . Most of these differences are conservative sequence changes (Supplementary Figure 2) and only one of these sequence changes occurs at the dimer interface. StTFE- β shares a sequence identity of 64% with the thiolase subunit of the PfTFE complex and the StTFE- β dimer superimposes well on the thiolase dimer of the PfTFE structure. The close sequence relationship between EcTFE- β and StTFE- β suggests that the StTFE- β thiolase is also a weak dimer. Although the thiolase dimer interface is extensive, also some other thiolases have been shown to be transient weak dimers (Kiema et al., 2019). In addition, the EcTFE- β chain is inactive. Reconstitution experiments show that the EcTFE tetramer is formed and, in this complex, the β -chain has regained its catalytic activity. This has also been observed for the PfTFE β -chain in similar reconstitution experiments with the PfTFE subunits (Ishikawa et al., 1997). Clearly, in both TFEs the formation of the catalytically competent thiolase dimer is favored when being complexed with the α -chain.

Substrate specificity of EcTFE

The wide substrate specificity range of acyl-CoA metabolizing enzymes is an intriguing property of these enzymes. EcTFE metabolizes short chain 2E-enoyl-CoA much more efficiently than medium and long chain 2E-enoyl-CoA substrates (Sah-Teli et al., 2019). A comparison of the enoyl tail binding pockets of the EcTFE ECH active site (Figure 7) shows that its binding pocket is rather narrow, due to the presence of the large Trp87 side chain. In *P. fragi* there is only one TFE, corresponding to EcTFE, having a glycine (Gly88) replacing Trp87, and studies of PfTFE suggest that the substrate specificity properties of PfTFE are possibly different from EcTFE, being that in these studies long chain substrates are better degraded than short chain substrates (Imamura et al., 1990). However, the properties of the α W87G-EcTFE variant show that a bulkier side chain at position 87 alone does not provide a rationale for the substrate specificity properties of EcTFE.

Possibly a conformational change, opening up the binding pocket, is triggered on binding of a substrate with an extended enoyl moiety, as has been reported for the mode of binding of octanoyl-CoA to the homologous monofunctional RnECH, (PDB code: 1DUB, PDB code: 2DUB).

Substrate channeling properties of EcTFE

Another interesting property of TFEs is the presence of substrate channeling. Enzyme kinetic experiments by Schulz and coworkers provide evidence for substrate channeling of EcTFE (Yang et al., 1985, Yang et al., 1986). For PfTFE such enzyme kinetic studies have not been reported but the high sequence and structural conservation between PfTFE and EcTFE suggest similar substrate channeling properties for PfTFE. The EcTFE tetrameric model obtained from the superpositions is shown in Figure 9. The tetramer is stabilized by the interactions between the α and β -chains, as well as between the two α -chains and between the two β -chains. The latter dimer corresponds to the classical thiolase dimer. The α - α interactions are formed by the protruding CH2A-CH2B helices of both chains. Such α - α interactions are not present in the MtTFE and HsTFE tetramers. This geometric feature divides the space between the α -dimer and the thiolase dimer in two bulk solvent accessible, interconnected cavities (Figure 9). The three active sites, catalyzing the last three subsequent β -oxidation reactions, line both cavities. Such a geometry of spatially close active sites facilitates substrate channeling (Ishikawa et al., 2004, Sweetlove and Fernie 2018). In addition, the surface of these cavities is predominantly positively charged, generating two positively charged reaction chambers (as visualized in Supplementary Figure 4b and Supplementary Video 1), favoring retention of the negatively charged acyl-CoA intermediates (Bernhardsgrütter et al., 2018). This cavity is also lined by the β -meander of the α -chain which points to the HAD active site as well as to the thiolase active site (Figure 9). At the tip of the β -meander there is Lys597, which would facilitate the transfer of the negatively charged acyl-CoA substrate between the HAD active site and the thiolase active site. In addition, Lys599 of this β -meander also points into this cavity. Lys597 and Lys599 are both conserved in PfTFE- α , however this loop is disordered in the respective crystal structure (PDB code: 1WDK). The importance of electrostatic interactions for substrate channeling properties of negatively charged intermediates has been shown experimentally in the studies of the bifunctional thymidylate synthase-dihydrofolate reductase and other enzyme systems (Anderson. 2017, Sweetlove and Fernie 2018) and in the diffusive binding studies of CoA-derivatives that move along positively charged polymers (Zheng et al., 2019).

ATP-mediated dissociation of EcTFE

In an attempt to understand how selectively only the EcTFE- α subunit was crystallized from the EcTFE solution, it was discovered that millimolar concentrations of ATP induce the disassembly of EcTFE into monomers of the α -chain, whereas the β -chain precipitates in these incubation mixtures. As observed from the crystal structure, ATP binds to the HAD part. However, preincubation of enzyme in the presence of millimolar concentrations of ATP does not negatively affect the catalytic ECH and HAD activities of the α -chain. Moreover, precipitation of the β -chain indicates direct interaction with ATP leading to the speculation that the disassembly of EcTFE is triggered by the binding of ATP to the β -chain. Interestingly, disassembly of the EcTFE complex in the presence of millimolar ATP concentrations is not observed for MtTFE. Noteworthy, EcTFE and MtTFE exhibit different tetrameric assembly and the substrate specificity of MtTFE has not yet been elucidated. The different behavior of these complexes in the presence of ATP could point to a different function of

these complexes. Possibly MtTFE is important for the metabolic degradation of specific fatty acid CoA conjugates, whereas EcTFE is critically important for the energy homeostasis of *E. coli*. It is well documented that fatty acid β -oxidation is regulated in various ways (Fujita et al., 2007). In addition to the transcriptional regulation, fatty acid β -oxidation is also affected by the levels of its products, being the ratio of NADH/NAD⁺, and the ratio of acetyl-CoA/CoA (Schulz. 1994, Lopaschuk et al., 1994). It is also observed that at higher ATP concentration fatty acid β -oxidation is inhibited in hepatic tissue by inhibiting the import of fatty acid transport across the mitochondrial membrane (Guzmán et al., 1996). The *in vitro* studies reported here suggest that fatty acid β -oxidation inhibition can also come from inhibition of the TFE- β activity at millimolar concentrations of ATP, at least in *E. coli*. It is interesting to note that the intracellular ATP concentration of *E. coli* is in the 1-5 millimolar range (Mempin et al., 2013). The ATP concentration seems to be very dynamic varying from cell-to-cell (Yaginuma et al., 2014). Inhibition of the catalytic activity of the thiolase subunit is also interesting considering that the TFE- β catalyzed reaction is the rate limiting step of fatty acid β -oxidation pathway (Li et al., 2018). Further research is required for better understanding of the possible role of the EcTFE- β inhibition by ATP in the regulatory mechanisms of the fatty acid β -oxidation.

Database Depositions

The atomic coordinates of the structure of EcTFE- α complexed with ATP have been deposited in the RCSB PDB database with the accession code 6TNM and will be released upon publication.

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Competing Interests

The authors declare that there are no competing interests for this study.

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Appendix A. Supplementary Material

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Tables

Table 1. Crystallization conditions, crystal handling and statistics of data collection, data processing and structure refinement

Dataset	With ATP bound (co-crystallization)
Crystallization	
Protein buffer	20 mM HEPES, 120 mM KCl, 2.5 mM DTT, pH 7.2
Protein concentration	5 mg mL ⁻¹
Crystallization well solution	100 mM Bis-Tris propane, pH 7.5, 25% w/v PEG 3550, 367 mM KNO ₃ and 25 mM ATP
Temperature	4 °C
Cryo protection	Reservoir solution with 20% v/v glycerol
Data collection	
Beam line	DLS I04
Detector	Eiger2 XE 16M
Wavelength (Å)	0.9795
Data processing	
Space group	C222 ₁
<i>a, b, c</i> (Å)	130.4, 214.1, 76.5
α, β, γ (°)	90.00, 90.00, 90.00
Resolution (Å)	65.19-2.95 (3.13-2.95)
R _{pim} (%)	10.8 (41.5)
CC _{1/2} (%)	98.7 (43.9)
I/σ(I)	5.9 (1.7)
Completeness (%)	100 (99.9)
Multiplicity	6.5 (6.7)
Observed reflections	149460 (24621)
Unique reflections	23039 (3661)
Wilson <i>B</i> -factor (Å ²)	53.0
Refinement	
Resolution (Å)	53.53-2.95
R _{work} /R _{free}	0.2300 / 0.2711
Number of atoms	
Protein atoms	5539
ATP	31
Glycerol	6
Nitrate	28
Waters	32
Average B-factor (Å²)	
Protein atoms	62.8
ATP	75.8
Glycerol	31.4
Nitrate	74.3

Waters	32.6
RMS deviations	
Bond lengths (Å)	0.0023
Bond angles (°)	0.6
Ramachandran plot	
Favored region (%)	96.0
Allowed region (%)	3.9
Outlier region (%)	0.1
PDB code	6TNM

Values in parentheses refer to the highest resolution shell.

Table 2. Hinge conformations of the ECH and HAD parts of TFE- α and MFE1

Hinge conformation of the ECH part					
	structure* (PDB code)	N-terminus of helix-3 (domain A)	middle of helix-10 (domain B)	distance (Å)	comment
EcTFE	6TNM (A)	Gly116	Phe293	16.6	
PfTFE	1WDK (A)	Gly117	Phe294	17.5	most open
	1WDK (B)	Gly117	Phe294	17.1	
HsTFE	6DV2 (G)	Gly148	Tyr343	16.9	
MtTFE	4B3J (A)	Gly116	Phe304	15.0	
	4B3J (B)	Gly116	Phe304	14.8	most closed
RnMFE1	5MGB (A)	Gly100	Phe271	17.3	
	5MGB (B)	Gly100	Phe271	16.6	
Hinge conformation of the HAD part					
	structure* (PDB code)	start of CH1, the pyrophosphate binding helix (domain C)	end of DH3, just before the substrate specificity loop (domain D)	distance (Å)	comment
EcTFE	6TNM (A)	Ile323	Val544	14.0	
PfTFE	1WDK (A)	Met325	Val544	14.4	
	1WDK (B)	Met325	Val544	14.6	most open
HsTFE	6DV2 (G)	Leu371	Val591	13.5	
MtTFE	4B3J (A)	Met335	Leu555	12.8	
	4B3J (B)	Met335	Leu555	11.8	
RnMFE1	5MGB (A)	Thr306	Ala524	10.2	most closed
	5MGB (B)	Thr306	Ala524	12.8	

* The chain identifier is specified in parenthesis.

Figure legends

Figure 1. The four enzymatic steps of the fatty acid β -oxidation cycle. The activities associated with TFE- α and TFE- β are highlighted.

Figure 2. Purification and characterization of the EcTFE-subunits. **a.** SEC profile and SDS-PAGE image of peak fractions of EcTFE, EcTFE- α and EcTFE- β . The full SDS-PAGE images are shown in supplementary Figure 5. **b.** CD spectra showing the secondary structural features of EcTFE, EcTFE- α and EcTFE- β . **c.** A graph showing the CD melting curves of EcTFE, EcTFE- α and EcTFE- β . **d.** SEC profiles of EcTFE and EcTFE reconstituted from its subunits. **e.** Bar chart showing specific activities of EcTFE- α , EcTFE- β and the reconstituted EcTFE complex relative to the purified EcTFE complex using 2*E*-butenoyl-CoA as substrates for ECH and HAD activities and acetoacetyl-CoA as substrate for KAT activity. The plotted values are relative with respect to the specific activity of EcTFE, which is arbitrary set to 100%.

Figure 3. Effect of ATP, ADP and NAD⁺ on EcTFE and its subunits. **a.** BN-PAGE image showing the dissociation of EcTFE complex upon preincubation with 5 mM ATP. MtTFE does not dissociate upon preincubation with 5 mM ATP. **b.** BN-PAGE image showing the effect of ATP and ADP on EcTFE- β . **c.** BN-PAGE image showing both EcTFE as well as MtTFE do not dissociate upon preincubation with 5 mM ADP. **d.** BN-PAGE image showing 5 mM ATP or ADP or NAD⁺ has no effect on EcTFE- α . **e.** CD spectra in the far UV region of EcTFE, EcTFE- α , EcTFE- β with and without preincubation with 5 mM ATP. Also, CD spectrum of EcTFE- β preincubated with 20 μ M ATP is shown.

Figure 4. Specific activities of EcTFE and its subunits. **a.** ECH specific activity. **b.** HAD specific activity. **c.** KAT specific activity. The ECH and HAD specific activities of EcTFE and EcTFE- α in the presence of 60 μ M 2*E*-butenoyl-CoA, after preincubation with 5 mM ATP or ADP or NAD⁺. The KAT activity of EcTFE with and without preincubation of 5 mM and 100 μ M ATP or ADP in the presence of 60 μ M acetoacetyl-CoA.

Figure 5. Domain organization of EcTFE- α . **a.** Domain nomenclature of EcTFE- α . The active site residues are also shown. **b.** Cartoon representation of EcTFE- α . The five subdomains of EcTFE- α are marked as A, B, C, D and E and colored differently. The C $_{\alpha}$ -C $_{\alpha}$ distance between Gly116 and Phe293 is shown, identifying the hinge conformation of the ECH part. Similarly, the C $_{\alpha}$ -C $_{\alpha}$ distance between Ile323 and Val544 identifies the hinge conformation of the HAD part. The residues used in these distance measurements are shown in sticks and the distances measured in Å are shown in purple dashed lines. The mode of binding of ATP (also shown in sticks) to the HAD part is also shown.

Figure 6. The mode of binding of ATP in the NAD⁺ binding site of the HAD part of EcTFE- α . The F_o-F_c omit electron density of ATP is shown as green mesh at a contour level of 3 σ . The H-bonding interactions between ATP and protein are highlighted in red dashed lines and the interacting residues are shown in purple sticks. The predicted mode of binding of NAD⁺ in EcTFE- α , obtained by superposing RnMFE1 complexed with NAD⁺ (PDB code: 3ZWC), is also shown. ATP is shown with cyan carbon atoms and NAD⁺ is shown with grey carbon atoms.

Figure 7. The acyl binding pocket of the ECH active site. **a.** EcTFE- α (PDB code: 6TNM, chain A), **b.** PfTFE (PDB code: 1WDK, chain A), **c.** HsTFE (PDB code: 6DV2, chain G), **d.** MtTFE (PDB code: 4B3J, chain A), **e.** RnMFE1 (PDB code: 3ZWC, chain A). The mode of binding of 3-hydroxydecanoyl-CoA (HSC, shown in sticks) in the RnMFE1 complex crystal structure (PDB code: 3ZWC, chain A) is used to compare the acyl binding pocket in the TFE structures. **f.** The mode of binding of acetoacetyl-CoA (CAA, shown in sticks) as bound in the complex of the monofunctional RnECH (PDB code: 1DUB, chain A). The estimated solvent accessible volumes of the acyl tail binding pocket in the ECH active sites are also shown.

Figure 8. Sequence and structure alignment of RnECH and EcTFE- α showing the properties of the loop-2/helix-2 region. **a.** Sequence alignment of the N-terminal part of RnECH and EcTFE- α highlighting the loop-2/helix-2 region, by a black line below the sequence. The two catalytic glutamate residues are identified with a blue star below the residues. **b.** Structural comparison of RnECH complexed with acetoacetyl-CoA (CAA shown in yellow stick) (PDB code: 1DUB; wheat cartoon) and complexed with octanoyl-CoA (CO8 shown in magenta stick) (PDB code: 2DUB; cyan cartoon). Only shown is the N-terminal region of the crotonase fold, including the residues listed in panel a. The residues Gly114, Lys115, Phe116, Leu117 and Ser118 as built in the 1DUB structure are labeled, which are disordered in the 2DUB structure. **c.** Structural comparison of EcTFE- α (pale green cartoon) with RnECH (1DUB, wheat cartoon and 2DUB, cyan cartoon). The EcTFE- α residues of the loop-2/helix-2 region (Ser85-Gln86-Trp87-Leu88-His89) which correspond to the residues being disordered in 2DUB structure are labeled.

Figure 9. The EcTFE tetramer model obtained by superposition of the EcTFE- α structure and the EcTFE- β dimer model on the PfTFE tetramer structure (PDB code: 1WDK). The tetramer structure is stabilized by α - β , as well as α - α and β - β interactions. The β -meander of EcTFE- α (black star) points to the HAD and the thiolase active sites that face cavity-1, which is highlighted as a light orange oval. Cavity-2 is highlighted as a light magenta oval. The horizontal thick arrow identifies the twofold axis of the tetramer. HSC (modelled from PDB code: 3ZWC), ATP (from the EcTFE- α structure) and CoA (modelled from PDB code: 1WDK) are shown as stick models, being bound, respectively, in the ECH, HAD and KAT active sites, that face cavity-1. The thin arrows connect the ECH and HAD active sites, as well as the HAD and KAT active sites. Similar view is also shown in stereo in the Supplementary Figure 4 and in Supplementary Video 1.

CRediT authorship contribution statement

R.V. conceptualized the work and acquired funding for the project leading to this publication. S.S. carried out cloning, purification, characterization, and structural studies. S.S. and M.H. purified and crystallized EcTFE/EcTFE- α . S.S. and R.S. cloned and purified α W87G-EcTFE variant for characterization. S. D. purified MtTFE. S.S. and R.V. determined and refined the crystal structure of EcTFE- α . W.S. synthesized the substrates necessary for enzymatic assays. S.S., R.K.W. and R.V. analyzed all the data and wrote the manuscript with the comments obtained from other authors.

Highlights

- EcTFE- α is a catalytically competent monomer while EcTFE- β is an active dimer only in the presence of EcTFE- α .
- Millimolar concentrations of ATP trigger the dissociation of the EcTFE complex and inactivate the EcTFE- β subunit.
- EcTFE- α selectively crystallizes from the EcTFE complex in the presence of ATP.
- In the obtained EcTFE- α structure ATP is bound at the HAD active site, close to the NAD⁺ binding pocket.
- The fatty acyl binding pocket of the ECH active site of EcTFE- α is narrower, which is in line with its preference for the shorter fatty acyl-CoA substrates.

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