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The multi-catalytic compartment of propionyl-CoA synthase sequesters a toxic metabolite

Iria Bernhardsgrütter^{#1}, Bastian Vögeli^{#1}, Tristan Wagner², Dominik M. Peter¹, Niña Socorro Cortina¹, Gert Bange^{3,4}, Sylvain Engilberge⁵, Eric Girard⁵, François Riobé⁶, Olivier Maury⁶, Seigo Shima², Jan Zarzycki¹, and Tobias J. Erb¹

¹Department of Biochemistry and Synthetic Metabolism; Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, D-35043 Marburg, Germany

²Microbial Protein Structure Group, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, D-35043 Marburg, Germany

³LOEWE Center for Synthetic Microbiology (Synmikro), Karl-von-Frisch-Str. 16, D-35043 Marburg, Germany

⁴Faculty of Chemistry, Philipps-University-Marburg, Hans-Meerwein-Strasse C07, D-35043 Marburg, Germany

⁵Institut de Biologie Structurale, University Grenoble Alpes, CEA, CNRS, F-38044 Grenoble, France

⁶Laboratoire de Chimie, École Normale Supérieure de Lyon, CNRS UMR 5182, Université Claude Bernard Lyon 1, 46 Allée d'Italie, F-69364 Lyon Cedex 07, France

[#] These authors contributed equally to this work.

Abstract

Cells need to cope with toxic or reactive intermediates formed during metabolism. One strategy is to sequester reactions that produce such intermediates within specialized compartments or tunnels connecting different active sites. Here we show that propionyl-CoA synthase (PCS), a ~400 kDa homodimer, three-domain fusion protein and the key enzyme of the 3-hydroxypropionate bi-cycle for CO₂-fixation, sequesters its reactive intermediate acrylyl-CoA. Structural analysis showed that PCS forms a multi-catalytic reaction chamber. Kinetic analysis suggests that access to the reaction chamber and catalysis are synchronized by interdomain communication. The reaction chamber of

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Corresponding author Correspondence and requests for materials should be addressed to Tobias J. Erb.

Data availability. The coordinates and structure factors of the crystal structure generated from this research are available at the Protein Data Bank under accession number 6EQO. All other relevant data are available in this article and its Supplementary Information files, or from the corresponding author upon request.

Author contributions I.B., B.V., D.M.P., J.Z., and T.J.E. conceived the project. I.B., B.V., T.W., J.Z., and T.J.E. designed and performed experiments and analyzed the data. E.G., F.R., and O.M. designed and prepared the phasing compound Tb-Xo4. I.B., B.V., S.E., E.G., T.W. and J.Z. collected X-ray data sets, T.W. and J.Z. solved crystal structures. E.G., G.B., and S.S. oversaw crystallography and SAXS experiments, provided equipment and beam-time. N.S.C. performed mass spectrometry and analyzed the data. I.B., B.V., T.W., J.Z., and T.J.E. wrote the manuscript with contributions from all other authors.

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PCS features three active sites and has a volume of only 33 nm³. As one of the smallest multi-reaction chambers described in biology, PCS could inspire the engineering of a new class of dynamically regulated nanoreactors.

Introduction

Biological systems face the challenging task of efficiently catalyzing hundreds to thousands of different chemical reactions in one “pot”, the cytoplasm. Diffusion is relatively fast compared to biochemical reactions, which leads to uniform concentrations of metabolites in the cytoplasm, in particular when considering the size and structural organization of microbial cells¹. The free diffusion of pathway intermediates can result in “cross-talk” between metabolic pathways and cause cross-inhibition, inactivation or even irreparable damage to metabolism, especially when the respective intermediates are unstable or reactive². Nature has evolved several strategies to ensure that problematic pathway intermediates are not released into the cytoplasm, but directly transferred to the next enzyme or active site. These strategies include encapsulation of intermediates in membrane- or protein-delimited organelles (compartmentalization), covalent attachment of intermediates to multi-domain enzyme complexes or carrier proteins, electrostatic guidance of intermediates from one active site to the next, or formation of direct intramolecular tunnels between two active sites³.

Here, we report on the enzyme reaction cascade from 3-hydroxypropionate to propionyl-CoA, which is the key sequence in the 3-hydroxypropionate bi-cycle for autotrophic CO₂ assimilation^{4,5}. The overall reaction sequence comprises three enzymatic steps, during which a highly reactive, toxic and unstable intermediate, acrylyl-CoA, is formed⁶. In autotrophic *Sulfolobales* the three reactions are catalyzed by individual enzymes^{7,8}. However, in several phyla (*e.g.* Proteobacteria and Chloroflexi, Supplementary Fig. 1) the three reactions are catalyzed by a fusion enzyme of about 1850 amino acids, that comprises three catalytic domains, the propionyl-CoA synthase (PCS; Fig. 1 and Supplementary Figure 2)⁴, suggesting that PCS specifically evolved as a fusion enzyme to overcome the free diffusion of reactive acrylyl-CoA. Here we show that the PCS from *Erythrobacter* sp. NAP1 (GenBank accession no. EAQ29651) is a multi-catalytic “nanoreactor” that features a central reaction chamber to sequester its reaction intermediates acrylyl-CoA during catalysis. Biochemical experiments show that access to the reaction chamber is dynamically controlled during the catalytic cycle and that catalysis is synchronized through interdomain communication.

Results

PCS sequesters the reactive intermediate acrylyl-CoA

To study the catalytic cycle of PCS from 3-hydroxypropionyl-CoA to propionyl-CoA (Fig. 1), we produced PCS from *Erythrobacter* sp. NAP1 (GenBank accession no. EAQ29651) and characterized the enzyme biochemically. When we followed the overall reaction of PCS, we could not detect acrylyl-CoA in the assay mixture, while the other intermediates 3-hydroxypropionyl-CoA and propionyl-CoA accumulated over time (Fig. 2a). Only when we

increased the concentration of the enzyme by two orders of magnitude, acrylyl-CoA became detectable, albeit at very low levels (Fig. 2b). The concentration of acrylyl-CoA corresponded to 1.8 ± 0.1 % of PCS monomers and stayed constant during steady-state. This demonstrated that acrylyl-CoA was formed *in situ* by the dehydratase domain, but presumably stayed sequestered within PCS, where it was quickly consumed by the reductase domain. Notably, when the reduction reaction was prevented (by omitting NADPH), acrylyl-CoA did not accumulate, while 3-hydroxypropionyl-CoA still did (Fig. 2c). This indicated that even when interrupting the catalytic sequence, acrylyl-CoA remained effectively sequestered within PCS. We could also show that externally added intermediates were not or only to a minor extent converted by the enzyme (Fig. 2d,e), as discussed in detail later in this manuscript.

PCS forms a multi-catalytic reaction chamber

To understand the structural basis of acrylyl-CoA sequestration in PCS, we solved the crystal structure of the enzyme in the presence of CoA, NADP⁺ and an ATP analog (phosphomethylphosphonic acid adenylate ester) using a new phasing compound⁹ (PDB 6EQO, Supplementary Table 1 and Supplementary Figs. 3 and 4). PCS forms a dimer of ~400 kDa around a central core of reductase domains (Fig. 1). The ligase and dehydratase domains extend to both sides, enclosing spherical compartments ('reaction chambers', Supplementary Fig. 5) that each feature three internal active sites (Fig. 3). The active sites are not connected through individual tunnels but all open into the central cavity of the reaction chamber. The surface of the central cavity is positively charged, which may help in retaining the CoA-ester intermediates during catalysis or even guide them between active sites as shown in the example of the malate dehydrogenase–citrate synthase cascade^{10–12}. Escape of intermediates from the reaction chamber is presumably disfavored by negative charges surrounding any small openings.

The inner diameter of the reaction chamber is between 3.5–5.5 nm with a total volume of 33 nm³. This volume is between three and six orders of magnitude smaller than that of bacterial microcompartments¹³ (14×10^3 to $\sim 10 \times 10^7$ nm³, calculated from inner diameters ranging from 30 to 600 nm)¹⁴, two orders of magnitude smaller than of described nanocompartments, such as encapsulins (5×10^3 nm³, calculated from inner diameter of 22 nm)¹⁵, and even half of that of proteasomes (59 and 84 nm³)¹⁶. Thus, PCS forms one of the smallest multi-catalytic reaction chambers observed in nature.

Structural elements in PCS mimic protomer contributions

The active sites of the acyl-CoA ligase, dehydratase and reductase domain of PCS align well with those of their corresponding lone-standing homologues (i.e., individual acyl-CoA ligase, dehydratase and reductase enzymes; Supplementary Figs. 6, 7 and 8). However, these lone-standing enzyme pendants are organized as homo-oligomers that require contributions from neighboring protomers in the oligomeric complex, whereas the individual domains of PCS seem to be organized as functional monomers within the enzyme^{18–20}. Notably, in PCS, the individual "monomeric" domains carry additional structural elements that apparently compensate (or "mimic") the essential contributions from the missing neighboring protomers in the lone-standing pendants (Supplementary Figs. 9, 10 and 11).

As an example, compared to lone-standing dehydratases, the dehydratase domain of PCS features additional helices (Supplementary Fig. 10) containing two highly conserved residues (Phe1220 and Lys1223, ref. 19) that are involved in stabilization of the CoA-ester. In the lone-standing dehydratases, these residues usually protrude from one protomeric subunit helix into the active site of a neighboring protomeric subunit of the homo-trimeric complex. Another example is the reductase domain of PCS that carries a structural extension compensating the part of the CoA binding site, which is provided by a neighboring protomeric subunit in lone-standing homo-tetrameric enoyl-CoA reductase/carboxylase homologues (Supplementary Fig. 11). Although the additional elements in the monomeric PCS domains structurally mimic the missing protomeric contributions from the lone-standing counterparts, they seem not to be related on the primary sequence level, which raises questions about the evolutionary history and importance of these additional structural elements.

In addition to these protomer-mimicking structural elements, the ligase domain of PCS carries structural extensions that are absent in any lone-standing acyl-CoA synthetase homologues (Supplementary Fig. 9), most prominently, an additional four-helix bundle. This four-helix bundle appears to be unique to PCS and is exclusively found in PCS homologues (based on BLASTP analysis). The four-helix bundle caps one side of the reaction chamber. Its absence would leave the reaction compartment wide open (Supplementary Fig. 14). Taken together, PCS is more than just a simple fusion of individual catalytic domains, but contains structural extensions that allow it to form a highly organized multi-functional enzyme compartment.

Access to the PCS reaction chamber is controlled

How is the sequence of reactions orchestrated within the compartment? The three enzyme reactions of PCS can be measured individually, when the appropriate substrates and cofactors are provided, demonstrating that all active sites are in principle accessible for their respective substrates⁴. We determined the kinetic parameters for the overall reaction of PCS as well as for each catalytic domain. While the ligase and the dehydratase domains had apparent turnover frequencies (k_{cat}) comparable to the overall reaction of PCS, the k_{cat} of the reductase domain was almost 30-fold higher (Supplementary Fig. 12, Supplementary Tables 2 and 3). This suggests that acrylyl-CoA is immediately consumed upon its formation *in situ*. To study whether externally provided intermediates can access the reaction chamber of PCS during steady-state, we performed an isotopic labeling competition experiment^{21,22}. When starting from 3-hydroxypropionate, PCS preferentially catalyzed the overall reaction. Externally added 3-hydroxypropionyl-CoA was unable to enter PCS under these conditions, while some externally added acrylyl-CoA was converted to propionyl-CoA. However, the amount of isotopically labeled propionyl-CoA corresponded well to the amount of acrylyl-CoA expected to be turned over in the pre-steady-state of PCS (i.e., within the first 0.6 s before 3-hydroxypropionyl-CoA is formed by the ligase domain; Fig. 2d,e and Supplementary Table 4). In other words: despite the high catalytic efficiency of the reductase domain, only a minor amount of externally added acrylyl-CoA was reduced by PCS in the steady-state. These results demonstrated that catalysis in PCS is consecutive, and that

internally produced 3-hydroxypropionyl-CoA and acrylyl-CoA are effectively channeled within the enzyme.

Interdomain communication regulates chamber access

Apparently, external acrylyl-CoA is prevented from entering the reaction chamber of PCS, indicating that the enzyme assumes a “closed state” during catalysis. Therefore, we wondered if any of the substrates (CoA, ATP, 3-hydroxypropionate) or products (AMP) would restrict access of exogenous acrylyl-CoA to the reductase domain during steady-state. Indeed, CoA had a strong effect on both the K_M and the k_{cat} of acrylyl-CoA consumption by the reductase domain. Most notably, binding of CoA to the ligase domain directly lowered activity of the reductase domain. We showed that Lys783 plays a crucial role in conferring this interdomain communication (Supplementary Figs. 12, 13 and Supplementary Table 2). The synchronization of domains in PCS was additionally supported by the fact that NADPH (the co-substrate of the third reaction) had a strong effect on the kinetic parameters of the first reaction. Notably, this effect was independent from an active reductase domain. NADPH had an ever stronger effect on the kinetic parameters of the ligase reaction when the cofactor was added to a dehydratase mutant of PCS (E1027Q variant), where the reductase domain is still functional but not provided with substrate due to the inactive dehydratase domain. These observations suggested that interdomain communication in PCS works in both directions (Supplementary Fig. 12 and Supplementary Table 2) and that the individual domains in PCS do not act independently from each other, but catalyze the three-step reaction sequence in a concerted fashion.

Taken together, our experiments demonstrated a functional coupling of the last reaction step in PCS to the first one and *vice versa*. Apparently, PCS undergoes synchronized conformational changes during catalysis allowing substrates and products to enter and leave the reaction chamber. The gatekeeper to the reaction chamber of PCS is presumably the ligase domain. Stand-alone CoA ligases undergo substantial conformational changes between “open” and “closed” states during catalysis^{18,23}. When we superposed a “closed-state” *Salmonella enterica* ligase (PDB 2P2F)¹⁸ with the PCS ligase domain, the structures aligned almost perfectly (rmsd of 0.932 Å over 441 Cα-atoms) with the exception of above mentioned additional four-helix bundle extension that is only present in PCS (Supplementary Figure 9). An “open-state” ligase of *Saccharomyces cerevisiae* (PDB 1RY2)²³ still aligned well to the PCS ligase domain with its N-terminal domain (rmsd of 0.955 Å over 374 Cα-atoms), while the C-terminal domain (~130 residues) appeared to be rotated outwards. Modelling a corresponding conformational change onto PCS resulted in the exposure of a “hole” that would provide access to the interior of an “open state” compartment (Supplementary Figure 14). Upon binding of CoA and formation of 3-hydroxypropionyl-CoA the ligase domain would switch back to the closed state, sealing the reaction chamber and sequestering the CoA-bound intermediates.

We performed limited proteolysis on PCS with trypsin in the absence and presence of different substrates and products to directly test for conformational changes of PCS during catalysis (Supplementary Figure 15). When incubated in the presence of CoA, which restricts access to the active site of the reductase and forces the enzyme in the “closed”

confirmation, PCS was fragmented within 90 min. In contrast, the simultaneous addition of 3-hydroxypropionate, NADP⁺ and ATP protected PCS from total proteolysis (Supplementary Figure 16). Peptide fragment analysis localized the changes in proteolysis to the flexible parts of the ligase domain that become solvent exposed in the “closed” formation and presumably buried in the “open” confirmation of the enzyme. In summary, limited proteolysis confirmed that the enzyme assumed different conformations depending on the presence of different substrates or products. Additional SAXS analyses of PCS in the presence of different substrates supported this conclusion (Supplementary Table 5).

Discussion

PCS, a key enzyme in the 3-hydroxypropionate bi-cycle for CO₂ fixation, catalyzes the three-step reaction sequence from 3-hydroxypropionate to propionyl-CoA. The enclosed reaction chamber, the observed interdomain communication and the proposed conformational changes suggest a highly complex and synchronized catalytic mechanism according to the following model (Fig. 4). First, the ligase forms 3-hydroxypropionyl-AMP from ATP and 3-hydroxypropionate in the open conformation. Binding of CoA then closes the reaction chamber, leading to the formation of 3-hydroxypropionyl-CoA inside the enzyme. CoA binding is facilitated by NADPH, which is consistent with the lowered $K_{M,CoA}$ observed upon NADPH addition and might ensure that the enzyme contains all necessary cofactors to catalyze the complete reaction sequence after closing the chamber. The formed 3-hydroxypropionyl-CoA is subsequently dehydrated to acrylyl-CoA within the closed reaction chamber, isolated from the external environment. The final reduction reaction triggers the re-opening of the reaction chamber for product release and prepares the enzyme for the next catalytic cycle. This mechanism fits well with the observation that the k_{cat} of the ligase reaction alone drops substantially in the presence of NADPH, indicating that NADPH stabilizes the closed conformation and limits the catalytic rate of the ligase domain.

Our experiments show that the three domains of PCS follow a synchronized reaction mechanism to sequester and channel the toxic intermediate acrylyl-CoA between active sites. Thus, PCS is not the simple fusion product of three individual enzymes, but represents a sophisticated three-dimensional arrangement of three different domains enclosing a central reaction chamber connecting all three active sites. The volume of the reaction chamber of PCS (33 nm³) is several orders of magnitude smaller than those of known bacterial micro- or nanocompartments. Usually proteinaceous compartments consist of self-assembling shell proteins that encapsulate their enzymes^{24–27}. In PCS, these elements are integrated into one polypeptide, which fulfills both the structural role of forming the reaction chamber, as well as the catalytic role of driving the multi-reaction sequence. This minimizes biosynthetic costs, because no additional proteins are required to build the compartment.

The three-reaction sequence in PCS is orchestrated through interdomain communication and conformational changes. Access to the reaction chamber is catalytically controlled by the ligase domain that is the only entry and exit site. This makes PCS a complex ‘nanoreactor’ and differentiates the enzyme from canonical channeling enzymes (e.g. tryptophan synthase^{28,29}, amidotransferases^{30,31}), which usually connect two active sites through a

narrow channel where conformational changes of single residues suffice to gate separate entry and exit sites^{30,32,33}. Compared to dimethylglycine oxidase (DMGO) that has also been described to possess an internal cavity and a single entry and exit funnel, PCS appears to be more complex, featuring a reaction chamber of three active sites and a more complex opening and closing mechanism^{34,35}.

PCS catalyzes three very fundamental chemical reactions in CoA-ester biochemistry and is able to retain free CoA-esters within its reaction chamber. Known CoA-ester enzyme cascades, such as polyketide, fatty acid or HMG-CoA synthases, require covalently attached CoA-intermediates or shared binding sites of the CoA moiety between two active sites to direct intermediates along a defined multi-reaction sequence^{36–38}. Compared to those examples, PCS employs an intriguingly simple design principle to catalyze a consecutive reaction sequence within a controlled environment. The natural example of a minimal self-assembling nanoreactor that is dynamically regulated could serve as a model for the engineering of spatially and temporally controlled reaction sequences^{39–44}, especially such that proceed via toxic, reactive or unstable intermediates.

Online Methods

a Chemicals

Chemicals were obtained from Sigma-Aldrich (Munich, Germany) and CARL ROTH GmbH (Karlsruhe, Germany). 3-hydroxypropionate was bought from TCI Deutschland GmbH (Eschborn, Germany). Coenzyme A was purchased from Roche Diagnostics. 1-¹³C-propionate sodium salt was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, USA). Biochemicals and materials for cloning and expression were obtained from Thermo Fisher Scientific (St. Leon-Rot, Germany), New England Biolabs GmbH (Frankfurt am Main, Germany) and Macherey-Nagel GmbH (Düren, Germany). Carbonic anhydrase was bought from MP Biomedicals (Illkirch, France). Primers or synthesized genes were obtained from Eurofins MWG GmbH (Ebersberg, Germany) or the DOE Joint Genome Institute (California, USA), respectively. Materials and equipments for protein purification were obtained from GE Healthcare (Freiburg, Germany), Bio Rad (Munich, Germany) or Merck Millipore GmbH (Schwalbach, Germany).

b Synthesis of 3-hydroxypropionyl-CoA, ¹³C-acrylyl-CoA and ¹³C-3-hydroxypropionyl-CoA

For the synthesis of unlabeled 3-hydroxypropionyl-CoA a previously described method using carbonyldiimidazole coupling of the precursor acid with coenzyme A was used⁴⁵. Unlabeled acrylyl-CoA was synthesized using a previously described mixed anhydride coupling via ethylchloroformate⁴⁵. ¹³C-acrylyl-CoA and ¹³C-3-hydroxypropionyl-CoA were synthesized in two steps from ¹³C-propionate. In the first step the CDI coupling method was adapted for the synthesis of ¹³C-propionyl-CoA by a protonation step. ¹³C-propionate (0.156 mmol, 4.8 eq.) was dissolved into 1 mL THF containing pTsOH (0.156 mmol, 4.8 eq.) for 15 min, the mixture was centrifuged and CDI (0.130 mmol, 4 eq.) added to the supernatant. The mixture was stirred at RT for 1 h. CoA (0.0325 mmol, 1 eq.) dissolved in 250 µL 0.5M NaHCO₃ was added and stirred for 1h. The mixture was

lyophilized, HPLC purified and again lyophilized. ^{13}C -acrylyl-CoA was synthesized enzymatically using the acyl-CoA oxidase Acx4 from *Arabidopsis thaliana* 46. A 1 mL assay contained 100 μL 1 M KHPO_4 200 μL 30 mM ^{13}C -propionyl-CoA and 600 μL 1mg/mL Acx4. The reaction was quenched after 1 h by adding 20 μL 50% formic acid and directly injected into the HPLC-MS for purification using a previously described purification protocol 45. In case of ^{13}C -3-hydroxypropionyl-CoA the assay contained additionally 50 μL of the dehydratase PhaJ from *Pseudomonas aeruginosa* for direct hydration of the *in situ* generated acrylyl-CoA.

c Bacterial strains and growth conditions

E. coli DH5 α (Thermo Scientific™) strains were used for cloning and grown in LB medium 47. For protein expression *E. coli* BL21-AI™ (Invitrogen) or Arctic-Express (DE3) RIL (Agilent Technologies) were grown in TB medium 48. Incubation temperature was 37°C. Antibiotics for selection purposes were used accordingly: 100 $\mu\text{g}/\text{ml}$ ampicillin, 15 $\mu\text{g}/\text{ml}$ gentamycin.

Cloning. All *in silico* cloning was performed with Clone Manager 9 (Scientific & Educational Software). For purification, preparation, cloning, transformation and amplification of DNA, standard protocols were used 49. Plasmid isolation and PCR product purification was performed with kits from Macherey Nagel (Düren, Germany) according to the manufacturer's protocols.

The gene encoding for PCS (GenBank accession no. EAQ29651) with an N-terminal 10x His tag was synthesized by the DOE Joint Genome Institute. The construct was cloned into the expression backbone pET-16b by restriction cloning, resulting in the plasmid pTE1005. Point mutants were generated by QuickChange® Site-Directed mutagenesis (Stratagene, La Jolla, USA). Following primers were used: forward primer (5'-CGT TTC GGT CAA CCA CAA ATC AAT CTT CGC-3') and reverse primer (5'-GCG AAG ATT GAT TTG TGG TTG ACC GAA ACG-3') for the E1027Q variant; forward primer (5'-CGG AAA TTT TTG GCA CAG CGC TGT GCA ATG CTT ATG AG-3') and reverse primer (5'-CTC ATA AGC ATT GCA CAG CGC TGT GCC AAA AAT TTC CG-3') for the H1769A variant; forward primer (5'-CCT CAC AGC CAG ATG GGT GTA ACT CC-3') and reverse primer (5'-GGA GTT ACA CCC ATC TGG CTG TGA GG-3') for the K783M variant.

d Expression and purification of PCS

PCS was expressed from the plasmid pTE1005 using *E. coli* ArcticExpress (DE3) RIL as expression host. The cells were transformed with the expression plasmid and plated on LB agar containing selective antibiotic and grown overnight. The colonies were used to inoculate 1 L TB medium. The expression culture was incubated at 37°C while shaking at 110 rpm until an OD_{600} of 0.7 – 0.9 was reached. The *E. coli* ArcticExpress (DE3) RIL culture was cooled down to 14°C before induction. Expression was induced by adding 0.25 mM IPTG. The culture was incubated for 16-20 h. The cells were harvested by centrifugation at 5000 $\times g$ for 10 min. The pellet was stored at -20°C, optionally. Cells were resuspended in a 1:3 ratio (w/w) in Buffer A (50 mM Tris-HCl pH 7.9, 500 mM NaCl) containing SIGMAFAST™ protease inhibitor (Sigma-Aldrich, Munich, Germany) and lysed

by ultrasonication. The lysate was cleared by ultracentrifugation at $50'000 \times g$ for 45 min at 4°C followed by filtration through a 0.45 µm syringe filter. The lysate was loaded onto a 1 mL His-Trap (GE Healthcare). Unspecifically bound proteins were washed off with 15 mL of 5 % Buffer B (50 mM Tris-HCl pH 7.9, 500 mM NaCl, 500 mM imidazole). To wash away the *E.coli* ArcticExpress (DE3) RIL Cpn60 chaperone, an additional wash step was performed with 15 mL removal buffer (50 mM Tris-HCl pH 7.5, 50 mM KCl, 20 mM MgCl₂, 5 mM ATP). PCS was eluted with 100% buffer B and applied to a pre-equilibrated HiLoad 16/60 200 pg superdex (GE Life Science) column (150 mM NaCl, 20 mM Tris HCl pH 7.9). The purity of the PCS was tested by SDS-PAGE (Supplementary Figure 17).

e Kinetic characterization of PCS

Spectrophotometric assays were set up to measure the activity of PCS. The assays were performed in 10 mm quartz cuvettes (Hellma Analytics) on a Cary-60 UV/Vis spectrometer (Agilent Technologies Inc. Santa Clara, CA, USA). The assay temperature was set to 30 °C. The parameters for the CoA ligase domain alone were measured using a coupling assay via myokinase (purified from ASKA JW1375), pyruvate kinase and lactate dehydrogenase (SigmaAldrich P02694). To probe the influence of acrylyl-CoA on the ligase reaction, the assay was repeated using the PCS E1027Q variant deficient in the enoyl-CoA hydratase reaction to avoid back reaction of acrylyl-CoA to 3-hydroxypropionyl-CoA. In this assay PCS and acrylyl-CoA were added to the reaction and incubated for 5 min at 30°C before starting the reaction with the addition of CoA. The effect of NADPH on the ligase reaction was also tested with the PCS E1027Q variant to avoid overall reaction. The reaction catalyzed by the dehydratase domain was assayed using the PCS H1769A variant that is deficient in the reductase reaction. Acrylyl-CoA formation was coupled to its reduction by a stand-alone reductase (Etr1p from *Saccharomyces cerevisiae*). The PCS reductase reaction was measured using the E1027Q variant that is deficient in the enoyl-CoA hydratase reaction to avoid the back reaction of acrylyl-CoA to 3-hydroxypropionyl-CoA. The assay was repeated in presence of different concentrations of free CoA. PCS and CoA were added to the reaction mixture and incubated for 10 min at 30°C before starting the reaction with the addition of acrylyl-CoA.

All reactions were measured by following the consumption of NADPH or NADH (ligase coupling assay) at 340 nm ($\epsilon_{\text{NAD(P)H}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) or at 365 nm ($\epsilon_{\text{NADH}} = 3.4 \text{ mM}^{-1} \text{ cm}^{-1}$). The detailed conditions for all assays can be found in Supplementary Table 2.

f Crystallization of PCS

All crystallization was performed at 18°C using the sitting drop method in 96-well 2-drop MRC Crystallization Plates in polystyrene (Molecular Dimensions, Suffolk, UK). Crystallization drops (1.4 – 2 µL) contained PCS at 10 mg/ml premixed with 2mM of CoA, NADP⁺ and phosphomethylphosphonic acid adenylate ester each mixed with reservoir solution in a 1:1 ratio. First thin needle-shaped crystals appeared after several weeks in 100 mM BisTris pH 6.5, 200 mM NaAc, 25 % (w/v) polyethylene glycol (PEG) 3350 supplemented with 3 % (w/v) trimethylamine N-oxide dihydrate as additive (condition 1). These crystals had a *C*₂ symmetry and the best resolution obtained was 2.7 Å, which was used for the final structure model. Increasing the additive trimethylamine N-oxide dihydrate

to 6 % still lead to crystal formation (condition 2) but exhibited strong twinning. Crystals could also be reproduced in the same condition replacing the additive with 100 mM D-(-)-fructose (condition 3) or 4 % (v/v) tert-butanol (condition 4). Crystals of the space group $P2_1 2_1 2$ were obtained in the same condition supplemented with 2 % (w/v) benzamidinium hydrochloride as additive (condition 5). Phasing was achieved by soaking crystals of condition 3 in 100 mM Xo4 9 for 4 minutes. All crystals were cryo-protected with the respective crystallization solution supplemented with 20 – 30 % ethylene glycol.

g X-ray crystallography analysis

Numerous heavy atom derivatives have been tested attempting to solve the structure of PCS such as: Potassium tetrachloroplatinate(II), organic mercury derivatives, 5-amino-2,4,6-triiodoisophthalic acid and lanthanide phasing compounds (NatX-ray SAS, Saint Martin d'Hères, France). These derivatives resulted in either very low occupancy of the heavy atoms or a substantial decrease in diffraction. A recently developed lanthanide complex, Xo4, containing a terbium ion 9 gave the best results with high anomalous signal not interfering with diffraction quality for short time soakings. We solved PCS in the $C2$ crystalline form using the single-wavelength anomalous scattering method. Datasets were collected at beamline Proxima-2A of the SOLEIL synchrotron (Paris, France) at the Tb LIII absorption edge (wavelength of 1.649165 Å) on two different crystals (condition 3, see above) soaked in Tb-Xo4. Merging provided a 3.45 Å resolution data set with high redundancy facilitating the location of 18 terbium sites using the SHELX 50 software and PHASER 51. After multiple cycles of phasing, electron density modification, and secondary structure building using AUTOSOL from the PHENIX package 52, the electron density quality was sufficient to build a model with Buccaneer from the CCP4 package 51. The initial model was then used as template for molecular replacement with a dataset of a native crystal (condition 1, see above) using PHASER-MR. The native dataset was collected (wavelength of 0.97625 Å) at beamline ID29 of the ESRF (Grenoble, France). Manual extension of the model was done using COOT 53. Several rounds of manual and automatic refinements were performed using COOT and PHENIX-Refine. The model (PDB 6EQO) structure was refined with Ramachandran statistics of 94.93 % favored, 4.82 % allowed, and 0.25 % outliers.

h Limited proteolysis

PCS at 0.5 mg/mL was forced into a supposedly closed or open state by adding 3 mM CoA or a combination of 3.4 mM ATP, 2 mM 3-hydroxypropionate and 2 mM NADP⁺, respectively. A zero time sample of 10 µL was taken. Trypsin (Promega, diluted in 25 mM Tris-HCl pH 7.5, 10 mM CaCl₂) was added in a protein:protease ratio of 200:1. Samples of 10 µL were taken at different time points. The sample was quenched with 10 µL 4x SDS buffer and heated at 90°C for 10 min. Samples were applied onto an SDS-PAGE gel. For peptide quantification, the limited proteolysis was repeated as described. However, the samples were quenched by adding PMSF protease inhibitor (dissolved in 2-propanol) to a final concentration of 1 mM. The propylation was performed overnight in the dark (at RT) in 100 mM HEPES buffer pH 7.5, 400 mM sodium cyanoborohydride and 5 % (v/v) acetone 54. The open state PCS samples were treated with unlabeled acetone while the closed state PCS samples were incubated with D6-labeled acetone. The reaction was stopped with 0.07 % TFA. Samples were concentrated and dried in a Speedvac. The open and closed state

samples of each time point were combined and purified over a C18 membrane (cut from Empore™ SPE disks). Peptides were eluted with 0.1 % trifluoroacetic acid (TFA) in 50 % acetonitrile (ACN). Samples were dried in the Speedvac. Peptides were resuspended in 50 µl 0.1 % TFA. 1 µl of the peptide sample was mixed with 1 µl solution of 3 g/L alpha-Cyano-4-hydroxycinnamic acid in 80 % ACN (v/v) containing 0.3 % TFA onto a MALDI plate. The dried spots were measured automatically for MS and MSMS in a MALDI TOF/TOF analyzer (Applied Biosystems/MDS Sciex, Framingham, MA, USA) and the 4800 Series Explorer Software.

i Time course assays

The time course assays with 0.1 µM PCS contained 0.8 mM CoA, 0.5 mM 3-hydroxypropionate, 0.8 mM ATP, 4 mM MgCl₂, 40 mM KCl, 50 mM KHCO₃ and 0.3 mM NADPH, if stated, in 100 mM potassium phosphate buffer pH 8. At specific time points 20 µL of the assay were quenched with 20 µL of 50 % formic acid. The samples were centrifuged at 17'000 × *g* and frozen in liquid nitrogen. Samples were immediately thawed before application to hrLC-MS. The time course assay with 10 µM PCS contained 5 mM CoA, 5 mM 3-hydroxypropionate, 5 mM ATP, 6 mM MgCl₂, 60 mM KCl and 2 mM NADPH in 100 mM Tris-HCl pH 7.8. At specific time points 10 µL of the assay were quenched with 10 µL acetonitrile and 10 % formic acid and directly injected into the hrLC-MS. Standard curves (0.05 µM to 500 µM) for quantification for 3-hydroxypropionyl-CoA, acrylyl-CoA and propionyl-CoA were prepared in the corresponding buffer conditions and treated in parallel to the samples. Acrylyl-CoA concentrations in PCS samples were too low for UV/Vis quantification, therefore MS based quantification using the extracted ion count of the standard curve was used. Acrylyl-CoA could only be detected via MS in the samples containing high amounts of PCS (acrylyl-CoA detection limit was 5nM).

j Isotopic labeling competition experiment

The competition contained 3 mM CoA, 2 mM 3-hydroxypropionate, 200 µM NADPH, 5 mM ATP, 7.5 mM Mg₂Cl, 60 mM KCl, 100 mM KHCO₃ and 100 mM Tris-HCl pH 7.8. For the competition either 100 µM ¹³C-3-hydroxypropionyl-CoA or 100 µM ¹³C-acrylyl-CoA was added. The assay was started with 2 µL of 1.28 mg/mL PCS wt and the reaction monitored photospectrometrically at 340 nm using a Cary-60 UV/Vis spectrometer (Agilent Technologies Inc. Santa Clara, CA, USA) at 30°C using quartz cuvettes (10-mm path-length; Hellma® (Germany)). The assay was quenched after a Abs of 0.36 that corresponds to a turnover of 60 µM. The isotopic pattern of the produced propionyl-CoA was analyzed by hrLC-MS.

k High resolution LC-MS (hrLC-MS)

3-hydroxypropionyl-CoA, acrylyl-CoA and propionyl-CoA were analyzed using an Agilent 6550 iFunnel Q-TOF LC-MS system equipped with an electrospray ionization source set to positive ionization mode through a 1290 Infinity UPLC (Agilent Technologies Inc. Santa Clara, CA, USA). Compounds were separated on a RP-18 column (50 mm x 2.1 mm, particle size 1.7 µm, Kinetex XB-C18, Phenomenex, Aschaffenburg, Germany) using a mobile phase system comprised of 50 mM ammonium formate pH 8.1 (A) and methanol (B). Chromatographic separation was carried out using the following gradient condition at a

flow rate of 250 µl/min: 0 min 0% B; 1 min 0% B, 3 min 2.5% B; 9 min 23% B; 14 min 80 %B; 16 min 80%; 17 min 0 % B; 18 min 0 % B.

Capillary voltage was set at 3.5 kV and nitrogen gas was used as nebulizing (20 psig), drying (13 l/min, 225 °C) and sheath gas (12 l/min, 400°C). The TOF was calibrated using an ESI-L Low Concentration Tuning Mix (Agilent Technologies Inc. Santa Clara, CA, USA) before measurement (residuals less than 2 ppm for five reference ions). MS data were acquired with a scan range of 750-1200 m/z.

CoA-thioesters were additionally detected by UV absorbance at 260 nm using a diode array detector (1290 Infinity II, Agilent Technologies Inc. Santa Clara, CA, USA)

LC-MS data were analyzed using MassHunter Qualitative Analysis software (Agilent).

I SAXS analysis

PCS was freshly purified as described above two days before SAXS analysis was performed. The protein was stored on ice until measurements. Gel filtration buffer for dilutions and blank measurements was treated equally. SAXS data were recorded at the European Synchrotron Radiation Facility (Grenoble, France) on beamline BM29. The protein was up-concentrated at the beamline. If stated, cofactors were added to the concentrated protein at following concentrations: 3-hydroxypropionate, 2mM; CoA, 3 mM; ATP, 3.4 mM. Two-fold dilution series (4 mg/mL to 0.125 mg/ml) were prepared by dilution with gel filtration buffer containing the corresponding cofactors. The different dilutions were measured to investigate sample quality. Sample storage and measurement temperature was set to 20°C. The ESRF BM29 online software was employed for primary data reduction. PrimusQt (version 4.8.1 55) was used for data analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Trifunctional PCS: Structure and reaction sequence.

Dimeric structure of PCS from *Erythrobacter* sp. NAP1 (PDB 6EQO). One protomer is depicted in cartoon and one in surface representation. The multi-domain organization is highlighted by different colors: orange, ligase domain; purple, dehydratase domain; cyan, reductase domain; blue sphere, N-terminus; red sphere, C-terminus. Schematic arrangement of the three domains and their individual reactions are shown using the same color code.

Figure 2. PCS sequesters the reactive intermediate acrylyl-CoA.

a, Time course of the overall reaction with 0.1 μM PCS, 800 μM CoA, 500 μM 3-hydroxypropionate, 800 μM ATP and 300 μM NADPH. Production of the 3-hydroxypropionyl-CoA intermediate (orange) and the final product propionyl-CoA (cyan) was observed. In contrast no free acrylyl-CoA was detectable. **b**, Time course of the reaction containing 10 μM PCS, 5 mM CoA, 5 mM 3-hydroxypropionate, 5 mM ATP and 5 mM NADPH. At these high enzyme concentrations acrylyl-CoA (purple) was detected at 0.18 μM during steady-state corresponding to 1.8% occupancy of reductase active sites. 3-Hydroxypropionyl-CoA and propionyl-CoA accumulate over time. **c**, as in **a**, but without NADPH. Again, formation of 3-hydroxypropionyl-CoA was observed, but not of free acrylyl-CoA. **d**, Isotopic labeling competition experiment containing unlabeled 3-hydroxypropionate and either ^{13}C -labeled 3-hydroxypropionyl-CoA (experiment 1) or acrylyl-CoA (experiment 2). The reaction was started by the addition of PCS. Products were analyzed by LC-MS (see Supplementary Table 4 for detailed assay conditions). **e**, Results of the isotopic labeling competition experiment. Only 0.8 ± 0.4 % of propionyl-CoA was produced from exogenous ^{13}C -labeled 3-hydroxypropionyl-CoA during steady state (experiment 1). Approximately every fifth propionyl-CoA (21 ± 15 %) was formed from exogenous ^{13}C -labeled acrylyl-CoA during steady-state (experiment 2). **a – c**, data of a representative single experiment. **e**, data mean $\pm$ s.d. ($n=3$).

Figure 3. Multi-catalytic reaction chamber of PCS.

Volume filling representation¹⁷ of the reaction chambers enclosed by PCS. The central catalytic reaction chamber of each protomer is formed through the contribution of all three domains. Orange, contribution of the ligase domain; purple, contribution of the dehydratase domain; cyan, contribution of the reductase domain. The close up shows a cross section through the reaction chamber. Electrostatic charge distribution is shown as a gradient from negatively charged (red) to positively charged (blue). The three active sites are well connected within the reaction chamber. Large positively charged patches may help in

retaining the CoA-ester intermediates inside during catalysis or even guide them between active sites. Negative charges around the small openings may also prevent leakage of the negatively charged CoA-derivatives. The PCS structure co-crystallized with CoA (no density), an ATP analog and NADP⁺ is depicted in cartoon showing the ligase domain in orange, the dehydratase domain in purple and the reductase domain in cyan. CoA binding sites have been modelled based on the superposition of the structures of lone-standing CoA ligase (PDB 2P2F)¹⁸, dehydratase (PDB 5JBX)¹⁹ and reductase (PDB 4A0S)²⁰ onto PCS (compare Supplementary Figs. 6, 7 and 8). Distances between the active sites have been determined by measuring the distance between sulfur atoms of modelled CoA moieties: ligase–dehydratase, 42.5 Å; dehydratase–reductase, 33.7 Å; ligase–reductase, 63.5 Å.

Figure 4. Proposed catalytic cycle of PCS.

In the open conformation, 3-hydroxypropionate (3OHP) and ATP are converted to 3-hydroxypropionyl-AMP (3OHP-AMP) through the ligase domain (orange). The binding of CoA induces closing of the enzyme and the formation of 3-hydroxypropionyl-CoA (3OHP-CoA). 3OHP-CoA is released into the reaction chamber, where it is converted by the dehydratase domain (purple) to acrylyl-CoA. Acrylyl-CoA then enters the active site of the reductase domain (cyan). Following the reduction of acrylyl-CoA to propionyl-CoA, the reaction chamber reopens to release propionyl-CoA, which leaves PCS ready for the next catalytic cycle.