New Options for Rational Biosynthetic Engineering of Novel Polyketide Drugs

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Many medically relevant molecules such as rifamycin, erythromycin, rapamycin or epothilone, are natural products of the polyketide type. These complex molecules are assembled from activated short chain carboxylic acid precursor molecules in a stepwise fashion. However, the structural variability of introduced sidechains was considered to be limited due to the dependence on typical cell metabolites such as malonyl-CoA acting as precursors. Recently, a newly discovered family of enzymes, the crotonyl-CoA carboxylase/reductases (CCR), has been shown to generate a wide variety of additional precursor molecules thus explaining the enormous observed chemical diversity of polyketides. Understanding the structure and mechanism of this enzyme class thus should pave the way for engineering novel drugs.

Terrestrial bacteria, such as Streptomyces and Myxobacteria, are a true treasure trove of medically relevant small molecules. Most of them are so-called polyketides, which are used e.g. as antibiotics (erythromycin) or as immunosuppressants (tacrolimus). These polyketides are formed stepwise from precursors by huge multimodular enzyme complexes called polyketide synthases (Wilkinson & Micklefield, 2007). This mechanism nicely resembles the assembly lines of car manufacturers (Weissman & Müller, 2008). The first module recognizes the first building block and covalently binds it to way stations in the assembly line. Next, the second module is loaded with a second building block and catalyzes the condensation of the two building blocks in a Claisen-type ester condensation. Subsequently, the intermediate is delivered further to the next module, which installs another building block to the growing chain. After several rounds of condensation and subsequent reductive steps, the last module releases the mature polyketide molecule.

In principle, altered and ideally pharmaceutically improved variants of known drugs or even completely novel substances can be generated by extending the spectrum of building blocks. However, the condensation chemistry employed...
requires a dicarboxylic acid for each carbon-carbon bond formation within the elongation step because decarboxylation serves as the energetic driving force of the reaction. In most cases the extender unit malonyl-CoA, derived from primary metabolism, is used, leading to a chain extension by two carbon atoms resulting in an intermediate not carrying a side chain (Fig. 1A). The extension with methylmalonyl-CoA, correspondingly, incorporates a methyl group as side chain. However, the structures of many described natural products cannot be explained by the incorporation of these two commonly available extender units (Fig. 1B). The formation of these side chains would require unusual building blocks such as ethylmalonyl-CoA, chloroethylmalonyl-CoA or hexylmalonyl-CoA.

Fig. 1. Reactions catalyzed by crotonyl-CoA carboxylase/reductases (CCRs).
A) CCRs generate a number of different building blocks for polyketide biosynthesis under consumption of NADPH and CO₂.
B) Production of unusual building blocks for the polyketide syntheses by means of reductive carboxylation and the installation in bacterial polyketide metabolites: (1) hexylmalonyl-CoA; (2) chlorocrotonyl-CoA; (3) 2-carboxyl-5-methylhexanoyl-CoA; (4) 2-carboxyl-4-methylhexanoyl-CoA; (5) 2-carboxyl-4-methylpentanoyl-CoA. PKS: polyketide synthase; NRPS: non-ribosomal peptide synthetase; Leu13: P450-epoxidase. Figure: HZI
However, no biochemical transformations were known that could yield such products from primary metabolites until the group of Georg Fuchs (Freiburg) recently described an unprecedented enzyme, which can catalyze the conversion of crotonyl-CoA to ethylmalonyl-CoA (Erb et al, 2007). Importantly, a number of homologues to the encoding gene were found in diverse secondary metabolite biosynthetic gene clusters (Erb et al, 2009) indicating that unusual side chains in polyketides may indeed be generated employing building blocks not stemming from primary metabolism, but produced by additional specific enzymes, which were named crotonyl-CoA carboxylase/reductase (CCRs) according to their first described representative. These enzymes utilize CoA esters of unsaturated fatty acids (e.g. 2-octenoyl-CoA) as substrates and catalyze a reductive carboxylation to generate the ready-to-use building blocks of polyketide synthases (2-carboxyloctanoyl-CoA / hexylmalonyl-CoA) (Fig. 1A) under consumption of NADPH and CO$_2$.

For a while it remained unclear how exactly these unusual reactions proceed and how the CCRs recognize their substrates. For this reason, and because of the structural variety of polyketides under study in our laboratories, we were inspired to perform structural and biochemical studies on the CinF enzyme from *Streptomyces* sp. JS360 (Quade et al, 2012). CinF takes part in the biosynthesis of cinnabaramides, polyketides that can be applied as potential fungicides by inhibiting the fungal proteasome. The structural characteristic of the cinnabaramides lies in their unusual hexyl side chain, which derives from incorporation of the unusual building block hexylmalonyl-CoA. We could indeed show that this building block is provided by CinF employing 2-octenoyl-CoA as its substrate by reductive carboxylation (Rachid et al, 2011). However, CinF is also able to recognize substrates containing a shorter side chain, such as crotonyl-CoA (butenoyl-CoA), while typical CCRs can only convert crotonyl-CoA and are inactive towards bulkier substrates such as 2-octenoyl-CoA.

We were able to solve the crystal structure of CinF in complex with its bound substrates 2-octenoyl-CoA and NADP at high resolution. Thorough analysis of the structure provided elegant and clear explanations of the findings described...
above. CinF forms a tetramer (dimer of dimers), with the flexible CoA part of the 2-octenoyl-CoA being situated in a furrow between two respective monomers (Fig. 2 and 3A, B). The well-defined 2-octenoyl part instead is accommodated in a hydrophobic pocket inside each monomer (Fig. 3C). Compared with the unpublished structure of a CCR homologue from primary metabolism, which can only utilize crotonyl-CoA as substrate, the hydrophobic pocket of CinF is distinctly larger (Fig. 4A). This observation can be attributed to two amino acid mutations occurring in the substrate binding pocket: In case of CinF, the two small amino acids Ala163 and Gly362 provide sufficient space for the accommodation of 2-octenoyl-CoA, whereas the two bulkier amino acids Ile171 and Phe370 at the corresponding positions of other primary CCRs restrict the hydrophobic pocket and prevent binding of substrates exhibiting a longer side chain. Both residues in CinF were mutated to the corresponding CCR residues. Biochemical characterization of the resulting enzyme variants showed a loss of activity towards 2-octenoyl-CoA; crotonyl-CoA, however, was still converted to ethyl-malonyl-CoA indicating that indeed these two positions define the substrate specificity of CCRs.

Another CCR involved in biosynthesis of the proteasome inhibitor salinosporamide, SalG, whose biochemical investigation was already conducted, is able to reductively carboxylate the unusual building block chlorocrotonyl-CoA. Therefore, a different substrate binding pocket of SalG was expected and based on its high sequence similarity to CinF, modeling of the architecture of its substrate binding pocket was feasible (Fig. 4B). It turned out that SalG also possesses an alanine in position 163, just like CinF, but an isoleucine in position 362. The latter occupies less space than phenylalanine in typical CCRs, but confines the binding pocket in comparison to the glycine in CinF, thus allowing for binding of medium-sized chlorocrotonyl-CoA.

The structure of CinF in complex with its bound substrates also gives insight into the unique reaction mechanism. NADP firmly binds to a loop between two domains of the protein, whereas the reactive nicotinamide group is placed close to the 2-octenoyl-CoA binding pocket (Fig. 3A, C). The double bond of

Fig. 3. Ligand binding by CinF. CinF is shown as a cartoon and in sticks; one chain is colored blue and the other one is yellow. NADP$^+$: green; 2-octenoyl-CoA: pink. The electron density is shown at 1.0 as gray mesh.
A) Interactions between NADP$^+$ and CinF showing the residues responsible for cofactor binding.
B) Binding of 2-octenoyl-CoA at the interface between these two monomers.
C) Close-up of the hydrophobic substrate binding pocket of the 2-octenoyl-chain.
D) A model showing how CO$_2$ (shown as cyan sticks) can be bound to the active center of CinF. Figure: HZI.
2-octenoyl-CoA, which is to be carboxylated during the reaction, is located in parallel orientation to the nicotinamide group of NADP. The reactive hydrogen atom of NADPH is thus located at a perfect position for double bond reduction. In the absence of CO₂ a slower side reaction was observed in the course of which the double bond is only reduced but not carboxylated. Despite intense efforts, we were not able to obtain a structure of CinF in complex with bound CO₂ and therefore had to rely on homology-based computer modeling to position CO₂ into the active site of CinF (Fig. 3D). In this structural model, CO₂ is able to form hydrogen bonds to Asn77 and Glu167, while hydrophobically interacting with Phe166. Interestingly, a very similar CO₂ binding pocket was described in the structure of the unrelated Rh-protein from *Nitrosomonas europaea* (Li et al, 2007). Indeed, mutations of the asparagine or glutamate led to the complete abolishment of the carboxylation activity of CinF, without abrogation of its reduction activity. CO₂ thus most likely is situated in parallel and in direct vicinity to the reactive double bond of 2-octenoyl-CoA within the binding pocket and opposite to the nicotinamide group of NADP. Consequently, product formation can proceed in a concerted fashion: The hydride of NADPH can attack the double bond of 2-octenoyl-CoA from above and the reaction can immediately proceed by attack of CO₂.

Our successful structure-function analysis of CinF now sets the stage to deliberately change the size of the substrate binding pocket of CCRs in a targeted fashion, in order to rationally modify their substrate specificity. It is thereby conceivable to generate novel and even unnatural building blocks for the polyketide biosyntheses. By incorporating these novel building blocks into polyketide backbones a number of variants of known structures with improved properties, e.g. higher effectiveness or fewer side effects, could be produced. However, it has to be taken into account that polyketide synthases will have to be biochemically fine-tuned to accept, incorporate and extend such novel extender units: a lengthy, but extremely worthwhile goal.

**Dirk Heinz**, born 1960, studied chemistry at the University of Freiburg; Dipl.-Chem. 1986; PhD at the Biocenter of the University of Basel, Dr. phil. nat. 1990; Postdoc at the University of Oregon (Eugene, USA) 1990-1993; Scientific Assistant at the University of Freiburg 1993-1998; Habilitation in Biochemistry at the University of Freiburg 1998; Junior Research Group Leader at the GBF 1998-2002; Head of Department of Structural Biology at the GBF 2002-2003; Head of Division of Structural Biology at the HZI 2003-2008; Honorary Professor
Rolf Müller studied pharmacy at the Bonn University and did his PhD at the Department of Pharmaceutical Biology, where he also worked as a postdoc. In 1996, he went to the Department of Chemistry at the University of Washington in Seattle, USA. At that time, he already began to investigate the production of antibiotics in bacteria and two years later came back to Germany as a junior group leader at the German Research Centre for Biotechnology (GBF, now HZI) in Braunschweig. In 2000, he completed his habilitation thesis at the Technical University Braunschweig about the biosynthesis of antibiotics in actinomycetes and myxobacteria. Since October 2003, Rolf Müller holds a chair as professor of pharmaceutical biotechnology at the Saarland University and in 2009 became the head of the Helmholtz-Institute for Pharmaceutical Research Saarland (HIPS). Furthermore, he heads the department of “Microbial Natural Products” (MINS) and co-founded the PharmBioTec GmbH in Saarbrücken. His research was rewarded with the Phoenix-Pharmacy Research Award on two occasions (2001, 2007), the DEHEMA Award for Natural Products Research (2002), the BioFuture Award of the Federal Ministry for Education and Research (BMBF, 2003) and the DEHEMA Award of the Max-Buchner Research Foundation (2010). In 2012 he became a member of the National Academy of Science and Engineering (acatech; Deutsche Akademie der Technikwissenschaften).

Publications


