Reversal of β-oxidative pathways for the microbial production of chemicals and polymer building blocks

Nicolai Kallscheuer, Tino Polen, Michael Bott, Jan Marienhagen*

Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, D-52425 Jülich, Germany

ABSTRACT

β-Oxidation is the ubiquitous metabolic strategy to break down fatty acids. In the course of this four-step process, two carbon atoms are liberated per cycle from the fatty acid chain in the form of acetyl-CoA. However, typical β-oxidative strategies are not restricted to monocarboxylic (fatty) acid degradation only, but can also be involved in the utilization of aromatic compounds, amino acids and dicarboxylic acids. Each enzymatic step of a typical β-oxidation cycle is reversible, offering the possibility to also take advantage of reversed metabolic pathways for applied purposes. In such cases, 3-oxoacyl-CoA thiolases, which catalyze the final chain-shortening step in the catabolic direction, mediate the condensation of an acyl-CoA starter molecule with acetyl-CoA in the anabolic direction. Subsequently, the carbonyl-group at C3 is stepwise reduced and dehydrated yielding a chain-elongated product. In the last years, several β-oxidation pathways have been studied in detail and reversal of these pathways already proved to be a promising strategy for the production of chemicals and polymer building blocks in several industrially relevant microorganisms. This review covers recent advancements in this field and discusses constraints and bottlenecks of this metabolic strategy in comparison to alternative production pathways.

1. Introduction

1.1. Fatty acid synthesis and β-oxidation

Fatty acid synthesis and β-oxidation are well-studied metabolic strategies for synthesis and degradation of fatty acids, respectively (Schulz, 1991; Wakil, 1961) (Fig. 1). The intermediates of both pathways are chemically identical, but different enzymes and cofactors are employed to prevent metabolic cross-talk. In eukaryotes, separation of both modules of fatty acid metabolism is also supported by localization in different compartments as β-oxidation is exclusively located in the mitochondria whereas fatty acid synthesis is located in the cytosol (Bartlett and Eaton, 2004).

Fatty acid synthesis is catalyzed by the fatty acid synthase complex (FAS), which keeps the growing fatty acid chain bound to an acyl carrier protein (ACP) during all steps of catalysis. The FAS exclusively uses malonyl-CoA, which is obtained from the carboxylation of acetyl-CoA, for chain elongation yielding 3-oxoacyl-ACP as first intermediate. For the subsequent reductions of 3-oxoacyl-ACP and enoyl-ACP, FAS employs NADPH as cofactor (Wakil, 1989) (Fig. 1). Multiple rounds of chain elongation and reduction of the carbonyl group at C3 in the growing carbon chain can be catalyzed by the FAS. Finally, the thioesterase domain of FAS cleaves the long-chain acyl-ACP releasing free fatty acids with a typical chain length of 16 or 18 carbon atoms.

In principal, β-oxidation is the reversal of fatty acid synthesis but involved enzymes are solely active on CoA-activated thioesters and do not convert the acyl-ACP thioesters of fatty acid synthesis. In the first step, an acyl-CoA synthetase is responsible for CoA-activation of fatty acids yielding the respective acyl-CoA thioesters (Groot et al., 1976) (Fig. 1). An acyl-CoA dehydrogenase uses FAD for the oxidation of acyl-CoA to *trans*-enoyl-CoA (Thorpe and Kim, 1995), which is then hydrated to 3-hydroxyacyl-CoA by an enoyl-CoA hydratase (Schomburg and Salzmann, 1990). Subsequently, a 3-hydroxyacyl-CoA dehydrogenase catalyzes the NAD^+-dependent oxidation of 3-hydroxyacyl-CoA to 3-oxoacyl-CoA (Schomburg and Stephan, 1995). The final chain shortening reaction is catalyzed by a 3-oxoacyl-CoA thiolase cleaving 3-oxoacyl-CoA to yield acetyl-CoA and a shortened $acyl_{n-2}$-CoA (Fig. 1), which can be further processed in subsequent β-oxidation cycles (Schomburg and Stephan, 1996). Released acetyl-CoA is metabolized in the citric acid cycle.

1.2. β-Oxidation-like strategies enable degradation of aliphatic and aromatic compounds

The mechanism of β-oxidation for the purpose of carbon chain-shortening is quite common in nature, not only in the context of fatty

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fatty acid synthesis
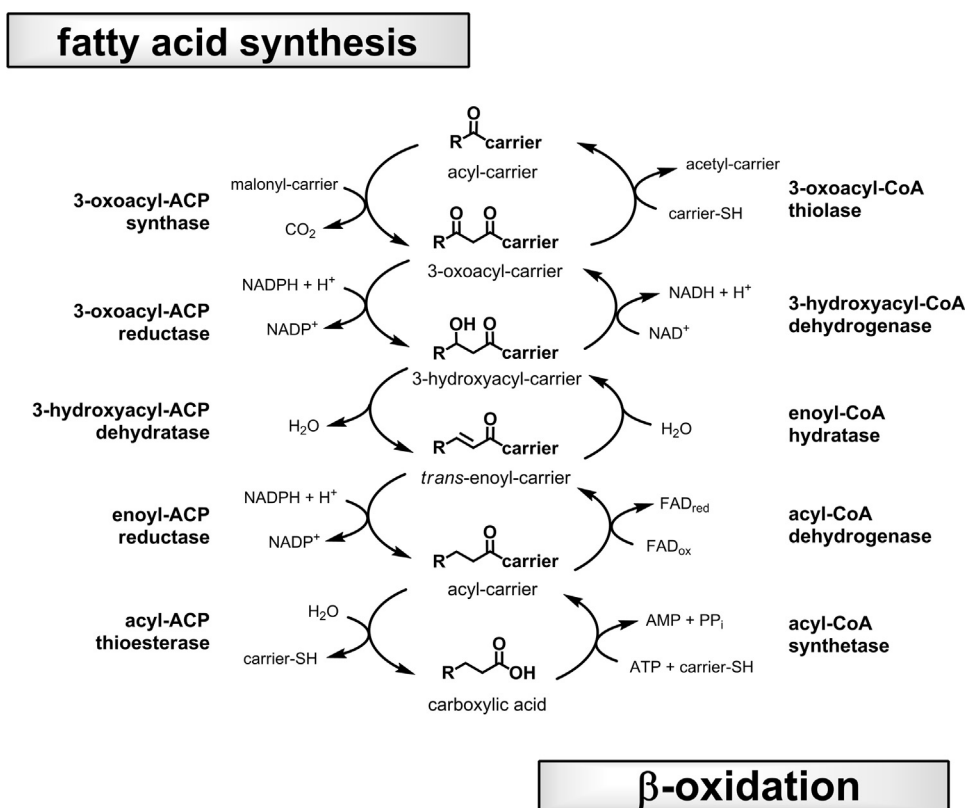
Fig. 1. Fatty acid synthesis and β-oxidation. One cycle of acyl chain elongation during the fatty acid synthesis pathway or acyl chain shortening by β-oxidation is shown. The acyl-carrier protein (ACP) and coenzyme A (CoA) are the respective carriers during fatty acid synthesis and β-oxidation, respectively. R represents a non-branched aliphatic carbon chain ($= (CH_2)_n$-CH_3).

β-oxidation

acid catabolism (Fig. 2A), but also for the degradation of aliphatic and aromatic compounds. Phenylacetic acid is an aromatic compound derived from ʟ-phenylalanine (Schneider et al., 1997). The aerobic degradation pathway for phenylacetic acid found in *Escherichia coli* leads to the formation of acetyl-CoA and 2,3-dehydroadipyl-CoA (a *trans*-enoyl-CoA) as intermediate, which undergoes β-oxidation including hydration, oxidation and thiolytic cleavage leading to succinyl-CoA and acetyl-CoA (Teufel et al., 2010) (Fig. 2B). Subsequently, both CoA-activated compounds are channeled into the citric acid cycle for further metabolization. In a similar manner, the dicarboxylic acid adipic acid is degraded in *Acinetobacter baylyi* (formerly *Acinetobacter sp.* ADP1) (Parke et al., 2001) (Fig. 2B). However, two preceding steps for CoA-activation of adipic acid and introduction of the double bond yielding 2,3-dehydroadipyl-CoA are required. Subsequent reaction steps for conversion of 2,3-dehydroadipyl-CoA to succinyl-CoA and acetyl-CoA are identical in *E. coli* and *A. baylyi*.

β-Oxidative chain-shortening reactions are also responsible for the aerobic microbial degradation of phenylpropanoids such as *p*-coumaric acid, which represent prominent plant-derived aromatic compounds (de Sousa, 2014) (Fig. 2C). A common CoA-dependent, β-oxidative degradation pathway for conversion of phenylpropanoids into benzoic acids was characterized in the soil-inhabiting bacteria *Rhodococcus jostii* RHA1, *Azoarcus sp.* EbN1 (inofficial designation "*Aromatoleum aromaticum*") and more recently also in *Corynebacterium glutamicum* (Kallscheuer et al., 2016b; Otani et al., 2014; Trautwein et al., 2012). In *Azoarcus* sp. EbN1 the final 3-oxoacyl-CoA thiolase-catalyzed chain-shortening step in the phenylpropanoid degradation pathway yields 4-hydroxybenzoyl-CoA and acetyl-CoA from *p*-coumaric acid (Trautwein et al., 2012). In contrast, in *R. jostii* and *C. glutamicum*, a ketohydrolase was found to catalyze the ultimate step yielding 4-hydroxybenzoic acid and acetyl-CoA (Kallscheuer et al., 2016b; Otani et al., 2014). 4-Hydroxybenzoic acid is hydroxylated to protocatechuic acid, which is then aerobically degraded by the β-ketoadipate pathway. The ultimate step in the β-ketoadipate pathway of *R. jostii* and *C. glutamicum* is the thiolytic cleavage of 3-oxoadipyl-CoA yielding succinyl-CoA and acetyl-

CoA (Fig. 2) (Patrauchan et al., 2005; Shen and Liu, 2005).

The anaerobic degradation of toluene in *Thauera aromatica* proceeds *via* (*R*)-2-benzylsuccinyl-CoA, which is converted to benzoyl-CoA and succinyl-CoA, also employing a β-oxidative route with the corresponding enoyl-CoA, 3-hydroxyacyl-CoA and 3-oxoacyl-CoA thioesters as intermediates (Leutwein and Heider, 2001) (Fig. 2D). By using one of two different branches of the central anaerobic benzoic acid degradation pathway benzoyl-CoA is further metabolized to pimeloyl-CoA, the CoA-thioester of the C_7 dicarboxylic acid pimelic acid (Harwood et al., 1998). Pimeloyl-CoA in turn is converted to glutaryl-CoA and acetyl-CoA *via* β-oxidation (Fig. 2E). This pathway was investigated in detail *e.g.* in *Rhodopseudomonas palustris* (Harrison and Harwood, 2005). Oxidation of glutaryl-CoA and decarboxylation of the pathway intermediate glutaconyl-CoA lead to the formation of crotonyl-CoA, which undergoes another round of β-oxidation yielding two additional molecules of acetyl-CoA (Harrison and Harwood, 2005) (Fig. 2F). Several strictly anaerobic bacteria such as *Acidaminococcus fermentans* follow the same β-oxidative pathway starting from crotonyl-CoA during the fermentation of ʟ-glutamate using the hydroxyglutarate pathway (Buckel, 2001) (Fig. 2F).

1.3. β-Oxidative pathways are mechanistically and thermodynamically reversible metabolic routes

3-Oxoacyl-CoA thiolases do not only catalyze the final thiolytic cleavage reaction during a typical β-oxidation, but also the reverse reaction: C-C bond formation following a Claisen condensation reaction mechanism (Haapalainen et al., 2006) (Fig. 3A). The simplest reaction is the condensation of two molecules of acetyl-CoA yielding acetoacetyl-CoA (a 3-oxoacyl-CoA). According to a thermodynamic analysis based on literature data, the reaction is endergonic as the change of Gibbs free energy $\Delta G°$ for acetoacetyl-CoA formation is positive ($\Delta G^{0'} = +29.6$ kJ/mol) (Dellomonaco et al., 2011) (Fig. 3B). This calculation is in line with the finding that the anabolic (3-oxoacyl-CoA-forming) reaction is thermodynamically unfavorable (Thompson et al.,