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Biochemistry of PUFA Double Bond Isomerases Producing Conjugated Linoleic Acid

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The biotransformation of linoleic acid (LA) into conjugated linoleic acid (CLA) by microorganisms is a potentially useful industrial process. In most cases, however, the identities of proteins involved and the details of enzymatic activity regulation are far from clear. Here we summarize available data on the reaction mechanisms of CLA-producing enzymes characterized until now, from Butyrivibrio fibrisolvens, Lactobacillus acidophilus, Ptilota filicina*, and* Propionibacterium acnes*. A general feature of enzymatic LA isomerization is the protein-assisted abstraction of an aliphatic hydrogen atom from position C-11, while the role of flavin as cofactor for the double bond activation in CLA-producing enzymes is also discussed with regard to the recently published three-dimensional structure of an isomerase from* P. acnes*. Combined data from structural studies, isotopic labeling experiments, and sequence comparison suggest that at least two different prototypical active site geometries occur among polyunsaturated fatty acid (PUFA) double bond isomerases.*

Introduction

Dietary PUFAs are potent growth inhibitors of ruminal bacteria.[1,2] In addition, linoleic acid (LA, 18:2$\Delta^{9Z,12Z}$; *x*:*y*Δ^{z} denotes a fatty acid with *x* carbons and *y* double bonds in position *z* counting from the carboxyl end) was demonstrated to inhibit the frequency of conjugation in Gram-negative bacteria.[3] Bacterial species of the gut microflora thus have access to a protective metabolic cascade known as biohydrogenation, which eliminates double bonds in PUFAs, ultimately producing saturated fatty acids. Early studies of biohydrogenation identified CLA as a first intermediate in the reduction of LA to stearic acid (18:0) by the ruminal bacterium *B. fibrisolvens*.[4] The discovery of anticarcinogenic properties of CLA[5] then stimulated, besides medical research, a strong interest in the bioconversion of PUFAs into their conjugated species.[6,7] Four PUFA double bond isomerases have been published to date, and in the following sections we summarize current data relating to their biochemical properties and reaction mechanisms.

Biochemistry of CLA-Producing Enzymes

To date, separate enzymatic activities producing either (9*Z*,11*E*)-CLA or (10*E*,12*Z*)-CLA have been found in numerous bacteria.[7–11] Several bacterial genes encoding for PUFA isomerases have been published, and in addition one eukaryotic isomerase has been isolated from the red alga *P. filicina*[12,13] (Table 1). However, the isolation of bacterial isomerases from native hosts remains challenging, likely because the isomerase activity has been shown to reside in the membrane fraction.[14,15] Probably for the same reason the overproduction of the active recombinant isomerase protein has been achieved only for PAI, which is relatively small and a soluble flavoprotein.[16]

To the best of our knowledge, the most detailed study of a microbial isomerase that produces (9*Z*,11*E*)-CLA (9,11-isomerase) was performed with a crude preparation of a membrane fraction from *B. fibrisolvens*.[4,14,17,18] Using sets of systematically designed substrates with varying head group polarity and double bond position, Kepler et al. demonstrated that the activity of this 9,11-isomerase was based on the presence of a free carboxylic group and a (9*Z*,12*Z*)-double bond system in the substrate. Fatty acids longer than 18 carbon atoms were not substrates. When a preparation harboring this enzyme was incubated with LA in the presence of $^{2}H_{2}O$, one ^{2}H atom was incorporated in the product (9*Z*,11*E*)-CLA in a pro-*R* stereo configuration (degree of labeling 88%), hinting at a reaction mechanism involving a prototropic shift. In addition, the kinetic isotope effect due to the breaking of a C–H bond at C-11 in LA was demonstrated by use of 11-dideuterio-LA as a substrate, with no loss of deuterium from the product being observed in this case. Therefore, both the protonation of the Δ12 double bond and the release of the intermediate LA carbanion from the active site were excluded as possible reaction pathways. Instead, Kepler et al. suggested a concerted mechanism for the LA isomerization, involving hydrogen removal from C-11 and stereospecific transfer (*R* configuration) of the solvent-derived proton by the enzyme to C-13 of the substrate (Figure 1 A).

In addition, several *Lactobacillus* species can convert LA into (9*Z*,11*E*)-CLA, but in contrast to the isomerase from *B. fibrisolvens*, varying amounts of (9*E*,11*E*)-CLA can be found in the reaction products.[15,19,20] Moreover, the cDNA sequence of the linoleate isomerase from *Lactobacillus acidophilus* has been

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Table 1. Identification and biochemical properties of PUFA double bond isomerases

Host, references	*Butyrivibrio fibrisolvens*[4,14,17,18]	*Lactobacillus acidophilus*[9,15]	*Propionibacterium acnes*[10,11,16,36]	*Ptilota filicina*[12,13,22]
Accession number	gene not identified	DQ23 9438	AX06 2088	AF35 4661
Substrate	18:2$\Delta^{9Z,12Z}$	18:2$\Delta^{9Z,12Z}$	18:2$\Delta^{9Z,12Z}$	20:5$^{\Delta5Z,8Z,11Z,14Z,17Z}$
Product	18:2$\Delta^{9Z,11E}$	18:2$\Delta^{9Z,11E}$ 18:2$\Delta^{9E,11E}$	18:2$\Delta^{10E,12Z}$	20:5$^{\Delta5Z,7Z,9Z,14Z,17Z}$
K_m [μM]	9.7	not determined	14	32.8
Protein properties	associated with membrane fraction	associated with membrane fraction	monomer, contains FAD	glycosylated, homodimer, contains flavin cofactor
Heterologous overexpression	no	no	*S. cerevisiae*, active *N. tabacum*, active *O. sativa*, active *E. coli*, active *L. lactis*, active	*E. coli*, inactive *A. thaliana*, active

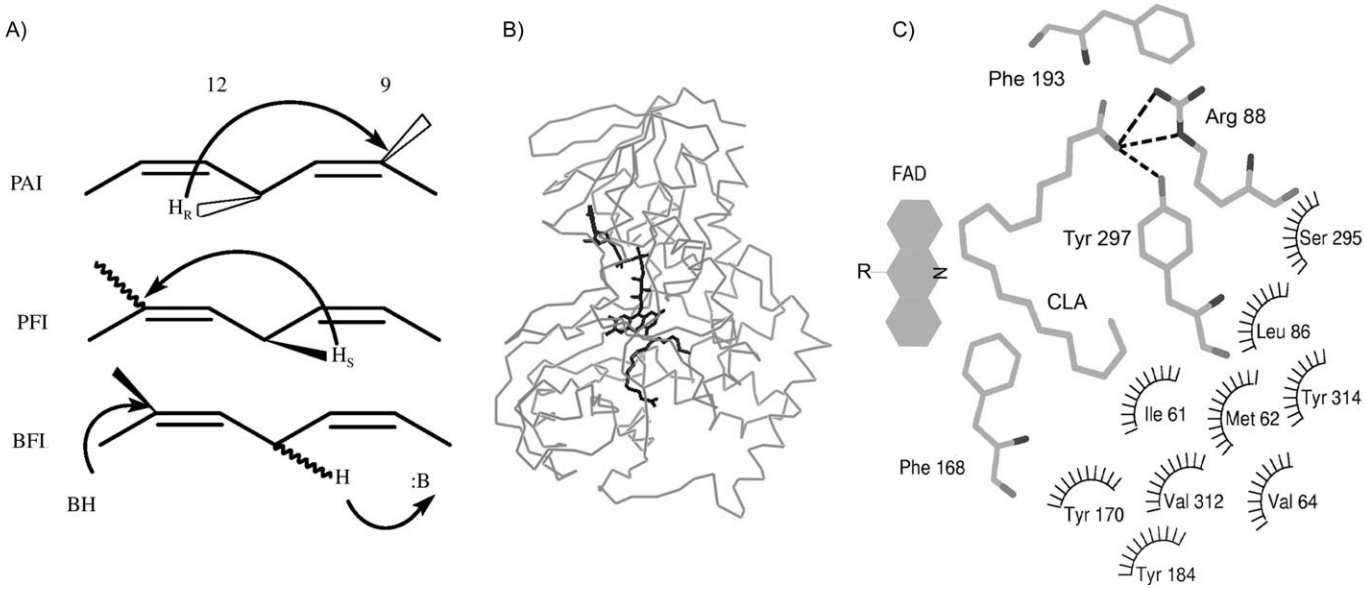

Figure 1. A) Hydrogen transfer by isomerases from *B. fibrisolvens*, *P. filicina*, and *P. acnes* (top to bottom). Only atoms C-8 to C-14 of LA are shown for clarity. Double bond positions are indicated by numbers. Waved lines indicate that the stereoconfiguration of hydrogen transfer has not been confirmed. For the isomerase from *P. acnes* the stereochemistry of the hydrogen transfer was predicted on the basis of structural data.[16] In the case of the isomerase from *P. filicina* the stereochemistry of hydrogen abstraction, but not its readdition, was elucidated by use of γ-linolenic acid labeled with deuterium at position C-11.[12] Only the site of hydrogen abstraction—not the stereochemistry—has been established for the isomerase from *B. fibrisolvens*.[18] Nevertheless, in this case the solvent-derived proton is incorporated in the product at C-13 in pro-*R* configuration.[18] B stands for a general base: an amino acid side chain or a water molecule, for example. B) Structure of the isomerase from *P. acnes* in complexation with (10*E*,12*Z*)-CLA (PDB ID 2BAB). The protein is shown as grey ribbon, whereas FAD and (10*E*,12*Z*)-CLA are shown as black sticks. C) Active site of the isomerase from *P. acnes* with the bound product (10*E*,12*Z*)-CLA. CLA and amino acids important for catalysis are shown as sticks. For clarity, FAD and amino acids forming hydrophobic contacts to CLA in the binding pocket are shown schematically.

submitted to public databases[9] (Table 1). However, until now its enzymatic activity has only been characterized with crude membrane preparations in which the enzyme was solubilized with β-octyl glucopyranoside, and besides the (9*Z*,11*E*)-CLA as main product, (9*E*,11*E*)-CLA and (9*Z*,11*Z*)-CLA were also detected in small amounts.[15] Whether these less abundant CLA isomers are side-products of this 9,11-isomerase specific to *Lactobacilli* or whether their formation is catalyzed by distinct proteins remains unclear.

Recent in vivo studies have linked CLA production with the formation of PUFA species containing hydroxy groups at C-10.[7,8,21] Apparently, a distinct pathway leading to CLA formation via (12*Z*)-10-hydroxyoctadec-12-enoic acid exists in some *Lactobacillus*, *Propionibacterium*, *Bifidobacterium*, and *Clostridium*-like bacteria of the human gut.[7] Several species of *Lactobacillus* have been reported to accumulate CLA isomers along with (12*Z*)-10-hydroxy- and (12*E*)-10-hydroxyoctadec-12-enoic acid during LA biotransformation under microaerobic conditions.[6,8] The requirement for low oxygen concentration and the suppression of culture growth by low levels of LA in the medium was similar to the conditions established for *B. fibrisolvens*.[14] Feeding of isolated (12*Z*)-10-hydroxyoctadec-12-

enoic acid to *L. acidophilus* cells or to mixed microbial cultures from human gut resulted in reduced levels of the substrate, accompanied by simultaneous production of (9*Z*,11*E*)-CLA.[7,8] It has to be noted, however, that the interconversion of these molecules was not shown directly with the isolated isomerase protein or by isotope labeling. Moreover, at the present moment the identities of enzymes and possible intermediates involved in the isomerization have not been confirmed.

The isomerase from *P. acnes*, the representative member of the 10,12-isomerase family, has been cloned, overproduced in *E. coli*, and characterized biochemically in terms of substrate specificity and the structures of resulting conjugated PUFAs.[11] The preferred substrate was LA, which was converted exclusively into (10*E*,12*Z*)-CLA, Unlike 9,11-isomerase from *B. fibrisolvens*, however, this 10,12-isomerase accepted long-chain PUFAs as substrates, and the formation of conjugated trienes from linolenic acid ($18{:}3\Delta^{9Z,12Z,15Z}$), arachidonic acid ($20{:}4^{\Delta 5Z,8Z,11Z,14Z}$), and docosahexaenoic acid ($22{:}6^{\Delta 4Z,7Z,10Z,13Z,16Z,19Z}$) was confirmed by HPLC, mass spectrometry, and NMR.

The only well studied example of a eukaryotic PUFA double bond isomerase is the one from *P. filicina,*[12] and to date no other double bond isomerases from higher organisms have been reported. In contrast with the microbial enzymes, this purified enzyme showed a clear preference for the ω-3 very long chain PUFAs such as eicosapentaenoic acid ($20{:}5^{\Delta 5Z,8Z,11Z,14Z,17Z}$) and docosahexaenoic acid ($22{:}6^{\Delta 4Z,7Z,10Z,13Z,16Z,19Z}$). The distinctive feature of its reaction behavior was the formation of a conjugated triene—that is, two double bonds of the PUFA were shifted by the enzyme when three or four double bonds were present in the substrate. However, the physiological role of the resulting conjugated trienoic PUFA in marine algae is yet not understood. γ-Linolenic acid ($18{:}3\Delta^{6Z,9Z,12Z}$) and dihomo-γ-linolenic acid ($20{:}3^{\Delta 8Z,11Z,14Z}$) were transformed by this isomerase into mixtures of conjugated diene and triene fatty acids, the latter products being predominant. In contrast, LA gave only a conjugated diene PUFA as a product, but the identity of this substance has not been established.[22] The proposed scheme for the first step of this reaction was similar to the mechanism elucidated for the isomerase from *B. fibrisolvens*, including the hydrogen abstraction at C-11 and its intramolecular transfer to C-13 with γ-linolenic acid as the substrate (Figure 1 A). Moreover, Wise et al. have suggested that the formation of the conjugated trienoic PUFA required an additional hydrogen elimination step followed by the concerted shift of two double bonds; at this stage one solvent-derived proton was incorporated at C-11 of arachidonic acid ($20{:}4\Delta^{5Z,8Z,11Z,14Z}$).[12]

Reaction Mechanisms of PUFA Double-Bond Isomerases

The early reaction models based on biochemical characterization of enzymes from *B. fibrisolvens* and *P. filicina* logically suggested the allylic shift mechanism in which the fatty acid intermediate is activated by hydrogen removal from C-11. However, these models clarified neither the natures of the hydrogen-removing groups in PUFA double bond isomerases nor the functional groups responsible for free carboxy group binding. However, the recently published structure of the isomerase from *P. acnes* in complexation with its product (10*E*,12*Z*)-CLA shed light on the atomic details of the reaction.[16] It turned out to be a flavoenzyme containing one noncovalently bound flavin adenine dinucleotide (FAD) moiety per protein unit (Figure 1 B). In the protein–(10*E*,12*Z*)-CLA complex the product FA adopted a strongly bent U-shaped conformation inside the highly hydrophobic substrate-binding channel (Figure 1 C). The carbon atom C-11 of LA was predicted to be the site of hydrogen abstraction and was positioned just 3.2 Å away from the redox-active nitrogen atom N-5 of FAD, immediately suggesting an intermediate FA oxidation by the latter during the catalytic cycle. Such a precise alignment of the substrate was achieved by hydrogen bonding between the free carboxylic group of the FA and two flexible residues at the entrance to the active site: Arg88 and Phe193 (Figure 1 C).

The mode of FA binding to the isomerase from *P. acnes* may allow some general conclusions to be drawn for both 9,11- and 10,12-isomerases. Because all published PUFA double bond isomerases have strictly preferred free FAs over esterified ones, it is likely that the free carboxylate group is recognized in a similar way by all group members. Moreover, the distance between the active site entrance and the N-5 atom of FAD might be very similar for all enzymes, based on the fact that C-11 of the FA substrate has been shown to be the hydrogen abstraction site in all three isomerases discussed so far.

In addition, the structural model allowed a two-step mechanism to be postulated for the isomerase reaction. The substrate LA is converted either into an intermediate carbocation or into a radical, by hydrogen or hydride anion removal, respectively, at position C-11 of the substrate, resulting in the reduced FAD. The hydrogen or the hydride anion, respectively, is then transferred back from FAD to the FA substrate at position C-9, due to the preferential stabilization of the mesomeric structure, with either a partial positive charge or an electron at C-9, by Phe168 (Figure 1 C).

In contrast to 9,11-isomerases, the reaction cycle of 10,12-isomerases does not result in proton exchange between the solvent and (10*E*,12*Z*)-CLA (A.L. and I.F., unpublished results, Figure 1 A). In view of the low sequence similarity between the three isomerases from *L. acidophilus*, *P. filicina,* and *P. acnes* (Figure 2), it is probable that, despite the conservation of the FAD binding motif, the overall geometries of the active sites in 9,11-isomerases and in 10,12-isomerases are different. The presence of water molecules or polar amino acid residues in the 9,11-isomerase active site might be a plausible explanation for proton exchange between the FA intermediate and the solvent. Thus, deuteration at C-13 could be mediated by the N–H bond at position N-5 in the reduced FAD, since tertiary amino groups easily exchange protons with the solvent. Recently, the incorporation of water-derived ^2H into (9*Z*,11*E*)-CLA at the C-13 position during the biotransformation of LA by mixed ruminal microorganisms has been demonstrated, with isotopic enrichment in CLA averaging at 26.8%, in comparison with 43.2% in water.[23] Together these data point towards the possibility of a

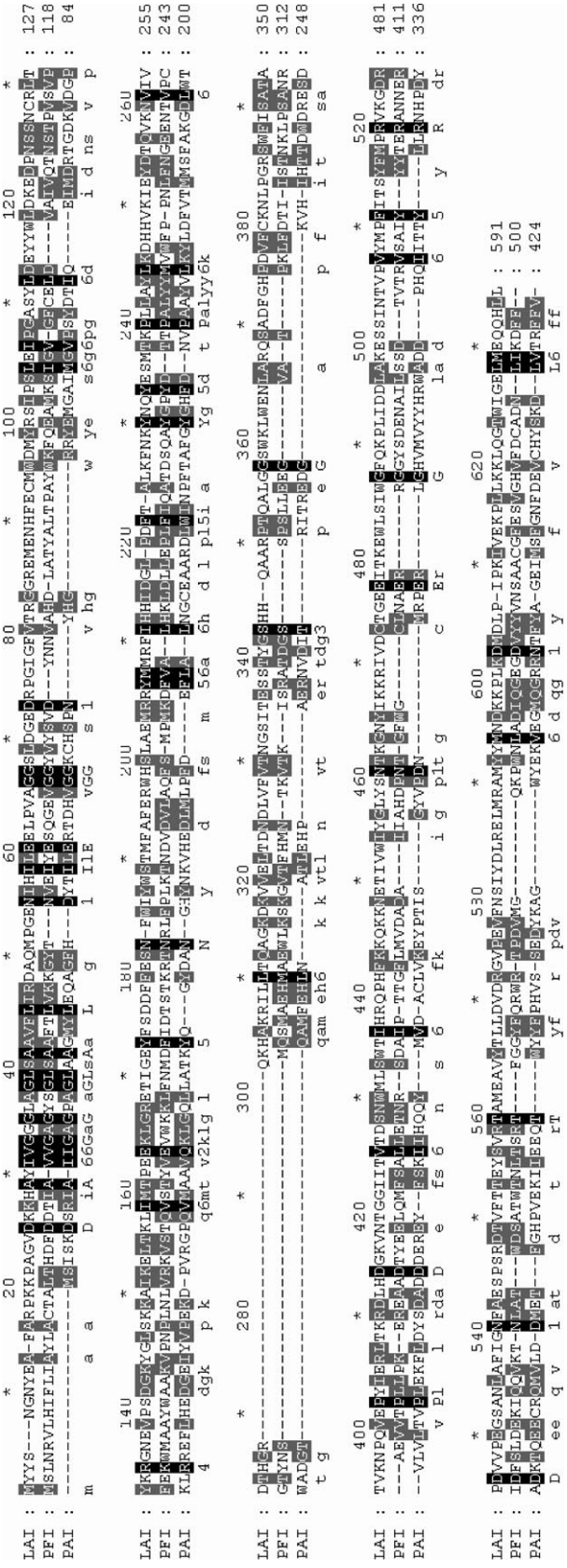

Figure 2. Sequence conservation between isomerase proteins from *L. acidophilus*, *P. filicina*, and *P. acnes*. Amino acid conservation between the three proteins is about 12% (counting relative to the sequence from *P. acnes*). The most strongly conserved region is their N-terminal FAD-binding domain, including the canonical GxGxxG(x)$_{18}$E nucleotide binding motif,[41] indicated by black rectangles.

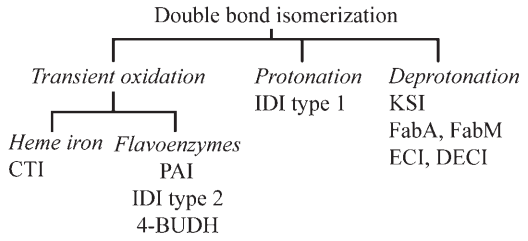

Figure 3. Classification of carbon-carbon double bond isomerases according to reaction mechanism. The list of enzymes was retrieved from http://expasy.org/enzyme/. Only the enzymes for which the reaction mechanism and/or structure was published were selected. The most common mode of double bond activation is deprotonation of the carbon atom next to the double bond by an acidic amino acid side chain, such as Glu or Asp. The pK_a of the allylic C–H is usually lowered by an adjacent keto or carboxy group, making an enzymatic proton abstraction relatively facile. This group consists of acyl-CoA- (enoyl-CoA isomerase (ECI), dienoyl-CoA isomerase (DECI) and acyl-ACP isomerases (FabA, FabM), as well as keto steroid isomerase (KSI). In contrast, protonation of the double bond by a Cys residue is a unique feature of an isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI) type 1 reaction.[26,42] A third option is double bond isomerization coupled to transient substrate oxidation, performed by cofactor-dependent enzymes of two types: 1) heme-containing *cis-trans* isomerase from *Pseudomonas sp.* (CTI), and 2) flavin-dependent 10,12-isomerase from *P. acnes*, isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2, and 4-hydroxy butyryl-CoA dehydratase (4-BUDH). All listed flavoenzymes contain FAD; for isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2 and 4-hydroxybutyryl-CoA dehydratase a radical mechanism of C–H bond cleavage, followed by substrate isomerization and readdition of hydrogen from FAD, has been demonstrated. For references see the text.

common reaction mechanism for all 9,11-isomerases. Unfortunately, until now none of these bacterial enzymes has been isolated as a pure protein or protein complex suitable for spectroscopic and structural analysis.

At first glance, the isomerization of LA to CLA is a simple chemical reaction; however, it requires an energetically demanding step to cleave a relatively inert C–H bond at C-11 in order to initiate the allylic shift of either the (9*Z*)- or the (12*Z*)-double bond. The allylic shift is driven by the negative free energy of conjugation, which in buta-1,3-diene is estimated to be about $-16.7\ kJ\ M^{-1}$, which makes the overall reaction irreversible. At least in the case of the isomerase from *P. acnes* the substrate activation and hydrogen transfer seems to be assisted by FAD, which might be also the case for other PUFA double bond isomerases, due to the conserved FAD binding domain (Figure 2).

Apart from PUFA double bond isomerases, the positional and geometrical isomerization of double bonds has also been studied in several other enzyme systems (Figure 3), including steroid delta isomerase,[24] isopentenyl diphosphate:dimethylallyl diphosphate isomerase types 1 and 2,[25–27] 4-hydroxybutyryl-CoA dehydratase,[28] (2*E*)-decenoyl-acyl-carrier-protein isomerase,[29] Δ3*Z*,Δ2*E*-enoyl-CoA isomerase,[30] Δ3,5-Δ2,4-dienoyl-CoA isomerase,[31] and *cis-trans* FA isomerase from *Pseudomonas* sp.[32] All listed enzymes, apart from 4-hydroxybutyryl-CoA dehydratase, modify lipophilic substrates and are involved in a variety of different metabolic pathways such as bacterial FA biosynthesis, FA β-oxidation, biosynthesis of isoprenoids, deg-

radation of steroids, and modification of plasma membrane lipids. Enzymatic double bond activation embraces three major mechanisms known from organic chemistry: 1) protonation of the double bond, 2) abstraction of the labile α-proton assisted by an electron-deficient keto or acyl group, and 3) oxidative cleavage of a C–H bond, which can generate either radical or carbocation intermediates. The latter option involving FAD and radical intermediates has been demonstrated for isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2 and 4-hydroxybutyryl-CoA dehydratase.[27,33,34] Notably, the structure of the PAI-(10*E*,12*Z*)-CLA complex also suggested a FAD-dependent mechanism of LA isomerization. Although there is no resemblance in terms of protein fold or substrate structure between isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2, 4-hydroxybutyryl-CoA dehydratase, and the 10,12-isomerase from *P. acnes*, all three enzymes likely employ FAD as a cofactor to cleave a stable C–H bond. It should be noted that the group of flavin-dependent carbon-carbon double bond isomerases so far includes only proteins from anaerobic bacteria. The absence of molecular oxygen in the environment enables a rich repertoire of carbon- and nitrogen-centered radical reactions in these microorganisms.[35] If the conservation of the FAD binding domain, hydrogen transfer data for the 9,11-isomerase from *B. fibrisolvens*, and the fact that most of the CLA producing bacteria are anaerobes or microaerophyles[4,7,23] are taken into account, it is tempting to speculate that flavin-dependent radical mechanisms may be involved in biosynthesis of all CLA isomers by bacteria.

Outlook

Steadily growing evidence relating to the beneficial health effects of CLA has resulted in a need for biotechnological production of pure CLA isomers. Four isomerase proteins, specifically producing either (9*Z*,11*E*)-CLA or (10*E*,12*Z*)-CLA, have been described to date, and several research groups have attempted to create transgenic plants producing pure CLA isomers through the introduction of the isomerases either from *P. acnes*[11,36] or from *P. filicina*.[13] These studies revealed that relatively small amounts of (10*E*,12*Z*)-CLA were accumulated in vivo only upon overexpression of isomerase gene from *P. acnes*. More precisely, (10*E*,12*Z*)-CLA made up about 0.3% and 0.9% of esterified FAs in the seeds of transgenic tobacco[11] and rice plants,[36] respectively. In the case of the isomerase from *P. filicina*, isomerase activity was detected in tissues of transgenic *A. thaliana* plants only by an in vitro assay, and no accumulation of any conjugated PUFA was detected in seed lipids.[13]

The structure of the FAD-containing isomerase from *P. acnes* provided the atomic model for the reaction mechanism. Despite the conservation of N-terminal FAD-binding motifs, comparison of kinetic parameters and proton transfer suggests a different organization of the active sites in 9,11- and 10,12-isomerases. Moreover, low sequence conservation between these two families restricts homology modeling efforts, although the isolation of individual 9,11-isomerases suitable for structural studies should resolve this intriguing question.

A major feature of the PUFA double bond isomerase reaction is the cleavage of inert C–H bonds in the absence of a strong oxidant. Conceptually, a similar reaction is catalyzed by lipoxygenases, which abstract bis-allylic hydrogen atoms in PUFAs, followed by the recombination of a FA radical with molecular oxygen.[37] Efficient transfer of atomic hydrogen by lipoxygenases is based on a quantum-mechanical tunneling effect, which has been demonstrated before.[38–40] Interestingly, flavin-dependent radical isomerization mechanisms have recently been demonstrated for two other unrelated bacterial flavoenzymes: isopentenyl diphosphate:dimethylallyl diphosphate isomerase type 2 and 4-hydroxybutyryl-CoA dehydratase. Apparently, in anaerobic bacteria the use of flavin radicals for transient substrate activation is a recurrent theme, which can be used for identification of new bacterial isomerases specific towards C–H bond cleavage.

Abbreviations

CLA: conjugated linoleic acid. FAD: flavin adenine dinucleotide. 9,11-Isomerase: PUFA double bond isomerase producing (9*Z*,11*E*)-CLA. 10,12-Isomerase: PUFA double bond isomerase producing (10*E*,12*Z*)-CLA. LA: linoleic acid. PUFA: polyunsaturated fatty acid.

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