Biochemical and Biophysical Research Communications 431 (2013) 675–679

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The role of C-terminal amino acid residues of a Δ^6-fatty acid desaturase from blackcurrant ☆

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ARTICLE INFO

Article history:
Received 11 January 2013
Available online 25 January 2013

Keywords:
Δ^6-Fatty acid desaturase
Δ^8-Sphingolipid desaturase
C-terminus
Amino acid residue
Enzyme catalysis activity

ABSTRACT

Δ^6-fatty acid desaturase is an important enzyme in the catalytic synthesis of polyunsaturated fatty acids. Using domain swapping and a site-directed mutagenesis strategy, we found that the region of the C-terminal 67 amino acid residues of Δ^6-fatty acid desaturase RnD6C from blackcurrant was essential for its catalytic activity and that seven different residues between RnD6C and RnD8A in that region were involved in the desaturase activity. Compared with RnD6C, the activity of the following mutations, V394A, K395I, F411L, S436P, VK3945AI and IS4356VP, was significantly decreased, whereas the activity of I417T was significantly increased. The amino acids N, T and Y in the last four residues also play a certain role in the desaturase activity.

1. Introduction

Delta fatty acid desaturases are nonheme iron-containing enzymes that catalytically create a double bond at a fixed position that is counted from the carboxyl end of a fatty acid [1]. Membrane-bound desaturases are divided into two groups: the acyl-CoA desaturases are mainly present in animals and fungi and the acyl-lipid desaturases exist in the plant cytoplasm [2]. The acyl-lipid desaturases contain an N-terminus of a fused cytochrome *b5*-domain and three histidine (His) boxes, $HX_{3-4}H$, $HX_{2-3}HH$ and $H/QX_{2-3}HH$, which have been proven to be essential for the function of the desaturases [3].

In all higher plants, Δ^8-sphingolipid desaturases (D8D) share a high identity with the Δ^6-fatty acid desaturases (D6D). Desaturated sphingolipids are ubiquitous membrane components, whereas Δ^6-desaturated polyunsaturated fatty acids are rare and only occur in a few higher species of plants [2]. Therefore, D8D may represent the ancestral form, and D6D may have evolved from D8D [2,4].

The Δ^6-acyl-lipid desaturases are the most widely studied membrane-bound plant fatty acid desaturases that convert substrate linoleic acid (LA; C18:2$\Delta^{9,12}$) and α-linolenic acid (ALA; C18:3$\Delta^{9,12,15}$) into γ-linolenic acid (GLA; C18:3$\Delta^{6,9,12}$) and octadecatetraenoic acid (OTA; C18:4$\Delta^{6,9,12,15}$), respectively. However, given the technical limitations in obtaining crystals from membrane proteins, information on the structure of membrane desaturases is scarce [5]. The functional characterization and structural data of D6D were obtained based on the heterologous expression and mutagenesis analysis of D6D in yeast.

Domain swapping and site-directed mutagenesis have been widely used to research the structure–function relationships of membrane-bound fatty acid desaturases because of the lack of three-dimensional information. A relatively hydrophobic region between the His boxes I and II of the ω3-fatty acid desaturase is regioselective [6]. Furthermore, seven conserved amino acid residues (aa) of the ω3-fatty acid desaturase of *Pichia pastoris*, within the His boxes I and II, are responsible for the regioselectivity switch with the Δ^{12}-fatty acid desaturase [5]. In addition, changing four relevant amino acid residues within a distance of five residues

Abbreviations: D8D, Δ^8-sphingolipid desaturase; D6D, Δ^6-fatty acid desaturase; LA, linoleic acid; ALA, α-linolenic acid; GLA, γ-linolenic acid; OTA, octadecatetraenoic acid; Cyt*b5*, cytochrome *b5*; GC–MS, gas chromatography–mass spectrometry; aa, amino acid residue(s).

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http://dx.doi.org/10.1016/j.bbrc.2013.01.062

from the His boxes of *Arabidopsis thaliana* FAD2 (Δ^{12}-desaturase) can convert this desaturase into a bifunctional desaturase/hydroxylase [7].

Domain swapping and site-directed mutagenesis were also widely used in studies of front-end desaturases. Domain deletion demonstrated that Cyt*b5* domain was essential for the desaturase activity, i.e., deleting either the first 112 or 146 residues of the Cyt*b5* domain of the borage Δ^6-fatty acid desaturase led to the loss of the desaturase activity [8]. Disruption of the his41 of the Cyt*b5* domain of borage D6D led to the failure to produce Δ^6-fatty acids [8]. In addition, replacing the first 113 residues of borage D6D with the residues from Arabidopsis D8D led to the decrease in the catalytic activity, further demonstrating the importance of the Cyt*b5* domain [9]. Researchers found that the mutation of the Cyt*b5*-like domain of the rat D6D could not recover its activity in the presence of other cytochrome *b5* proteins, and the free endoplasmic reticulum cytochrome *b5* also played a role in the D6D activity [10].

The three conserved His boxes and the residues near them have been widely investigated. Previous reports indicate that the variant glutamine in the third histidine box is essential for the D6D activity and that histidine could not substitute for the glutamine [9,11]. A mutational study of the conserved amino acid residues of Spirulina D6D demonstrated that H313 was involved in regioselectivity, i.e., changing H313 to R313 switched the D6D to Δ15 desaturase; that the three His boxes and H313, H315, D138 and E140 were likely to be providers of the catalytic Fe center; and that W294 might take part in forming part of the substrate-binding pocket [12]. The S213 and K218 that are adjacent close to the His box II of *Mucor rouxii* D6D were involved in the substrate recognition [13].

The functions of the C-terminal region of the membrane-bound Δ^{12}-fatty acid desaturase and Δ^{15}-fatty acid desaturase have previously been investigated [14,15]. The results showed that the carboxyl terminus of the desaturases had an important function in determining specific regiochemical and/or substrate characteristics. With regard to the D6D and D8D family, only one report showed that the C-terminal region might have an important role in the lipid head group recognition [4,5], but the report lacked detailed information. Blackcurrant Δ^6-fatty acid desaturase RnD6C and Δ^8-sphingolipid desaturase RnD8A have a high sequence similarity and a close phylogenetic relationship [16]. Therefore, these desaturases are suitable materials to study the relationship of functional region and the regioselectivity of D6D and D8D. In this study, using domain swapping and site-directed mutagenesis methods, we found that the region of the C-terminal 67 aa of D6D was essential and could not be deleted and that several amino acid residues in that region were involved in the D6D catalytic activity. The D6D activity of I417T was significantly increased to 132.6 ± 15.6% of RnD6C. However the D6D activity of VK3945AI was significantly decreased to 36.8 ± 3.01% of RnD6C.

2. Materials and methods

2.1. Chemicals and materials

The LA, ALA, GLA and heptadecanoic acid were obtained from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). The Pfu DNA polymerase was obtained from Transgen (Beijing, China). Restriction enzymes were obtained from Takara (Dalian, China). The vector pYES2 and yeast strain INV Sc 1 were obtained from Invitrogen (Paisley, UK).

2.2. Preparation of deletion and fusion mutations of RnD6C

The templates for the deletion and fusion mutation construct were the plasmid pyes-RnD6C and pyes-RnD8A [16] cloned into the *Kpn* I-*Sac* I sites of the plasmid pYES2 (Invitrogen, Paisley, UK). The primers used for cloning the deletion enzyme were CL and C381R (Table 1). By modifying the sequences of the fusion protein, we amplified the PCR fragments of the two different desaturases sharing common sequences at their ends using primers CL, CA381R1 (the PCR fragments was named Fus-CL), CA381L2, and AR (the PCR fragment was named Fus-AR) (Table 1). In the subsequent reaction, Fus-CL and Fus-AR were used as the template, and CL and AR were used as primers. Fus-CL and Fus-AR formed an overlap by hybridizing to each other via the common sequences at their ends. Extension of this overlap by the Pfu DNA polymerase (Transgen, Beijing, China) yielded a recombinant molecule. The temperature profile for a typical overlap extension PCR reaction was 94 °C for 3 min, 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min for 35 cycles, and 72 °C for 10 min.

2.3. Preparation of site-directed mutations of RnD6C

The template for site-directed mutations was the plasmid pyes-RnD6C [16]. The preparation of the site-directed mutations of *RnD6C* was performed using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Canada). The primers used in the mutation preparation are listed in Table 2. The PCR reaction temperature profile was 94 °C for 3 min, 94 °C for 30 s, 55 °C for 40 s and 72 °C for 7 min for 17 cycles. The mutations were amplified in *Eschershia coli*, sequenced and then transformed into yeast INV Sc 1.

The four mutations of C-terminal amino acid residues were prepared using primers listed in Table 3 based on RnD6C sequence. The temperature profile for the PCR reaction was 94 °C for 3 min, 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min for 35 cycles, and 72 °C for 10 min.

2.4. Yeast expression plasmid construction and transformation

2.4.1. Characterization of genes with Δ6-desaturase function in *Saccharomyces cerevisiae*

2.4.1.1. Fatty acid analysis. The methods for these three parts are previously described elsewhere [16].

3. Results

3.1. Construction of chimeric genes

Overlap extension PCR method for chimera construction allows the introduction of junction or mutation sites into the chimeric enzymes. There are only 8 different amino acid residue sites in the C-terminus (after His III) of RnD6C and RnD8A: K389T, VK394, 5AI, F411L, I417T, IS435, 6VP and Y447H (the letter to the left of the number represents the amino acid residues in RnD6C, the number represents the site of the different amino acid residue and the letter on the right of the number represents the amino acid residues in RnD8A). We deleted the C-terminal 67 aa of RnD6C and named it RnD6C381 (Fig. 1A); we fused the C-terminal 67 aa of RnD8A into N-terminal 381 aa of RnD6C and named it RnD6CA381 (Fig. 1A); we exchanged nine sites of RnD8A into RnD6C and named them individually as K389T, V394A, K395I, F411L, I417T, S436P, Y447H, VK3945AI and IS4356VP (Fig. 1B).

3.2. Functional characterization of the mutations

For functional identification, the mutated and the wild-type desaturases were expressed in *S. cerevisiae* strain INVScI, and the recombinant yeast cells were analyzed for fatty acids. Yeast was also transformed with an empty vector pYES2 as a negative con-

Table 1
Primers used for construction of the C-terminal deletion and fusion mutations of *RnD6C*.

Primers	Sequence (5′–3′)	Used for making fusion proteins
CL	ATGGGTGAAAATGGAAGGAAG	RnD6C381
C381R	TCATCTTGGGAACAAATGATGC	
CL	ATGGGTGAAAATGGAAGGAAG	RnD6CA381
CA381R1	CTAAGCTGGCATCGAGGCAATCTTGGGAACAAATGATGC	
CA381L2	GCATCATTTGTTCCCAAGATTGCCTCGATGCCAGCTTAG	
AR	TCAGCCATGGGTGTTGAC	

Table 2
Primers used for construction of the C-terminal site-directed mutations of *RnD6C*.

Primers	Sequence (5′–3′)	Used for making fusion proteins
ISVPL	GACCTATCCAACCCTGTTCCGAAGAATTTGCTATGGGAAG	IS435, 6VP
ISVPR	CTTCCCATAGCAAATTCTTCGGAACAGGGTTGGATAGGTC	
SpL	GACCTATCCAACCCTATTCCGAAGAATTTGC	S436P
SPR	GCAAATTCTTCGGAATAGGGTTGGATAGGTC	
ITL	GAGGCCAATGTTGCAACCATTAAGACGTTGAGGAC	I417T
ITR	GTCCTCAACG TCTTAATGGT TGCAACATTG GCCTC	
FLL	GGAGTTTATCATTTTTGGAGGCCAATGTTGCAATC	F411L
FLR	GATTGCAACA TTGGCCTCCA AAAATGATAA ACTCC	
VKAIL	GAAGGTCTCTCCTTTTGCGATAGAGCTTTGCAAGAAAC	VK394.5AI
VKAIR	GTTTCTTGCAAAGCTCTATCGCAAAAGGAGAGACCTTC	
VAL	GAAGGTCTCTCCTTTTGCGAAAGAGCTTTGCAAG	V394A
VAR	CTTGCAAAGC TCTTTCGCAA AAGGAGAGAC CTTC	
KIL	GAAGGTCTCTCCTTTTGTGATAGAGCTTTGCAAG	K395I
KIR	CTTGCAAAGC TCTATCACAA AAGGAGAGAC CTTC	
KTL	CGATGCCAGCTTAGGACGGTCTCTCCTTTTGTGAAAG	K389T
KTR	CTTTCACAAA GGAGAGACCGTCCTAAGCTGGCATCG	
nthL	GCTGTCAACA CCCATGGCTG AGAGCTC	Y447H
nthR	GAGCTCTCAGCCATGGGTGTTGACAGC	

Table 3
Primers used for construction of the C-terminal four amino acid residues site-directed mutations of *RnD6C*.

Primers	Sequence (5′–3′)	Used for making fusion proteins
NTHL	GCTGTCAACA CCCATGGCTG AGAGCTC	NTHG
NTHR	GAGCTCTCAGCCATGGGTGTTGACAGC	
HTHL	GCTGTCCACA CCCATGGCTG AGAGCTC	HTHG
HTHR	GAGCTCTCAGCCATGGGTGTGGACAGC	
NAHL	GCTGTCAACG CCCATGGCTG AGAGCTC	NAHG
NAHR	GAGCTCTCAGCCATGGGCGTTGACAGC	
NSHR	GCTGTCAACT CCCATGGCTG AGAGCTC	NSHG
NSHL	GAGCTCTCAGCCATGGGAGTTGACAGC	
ATHL	GCTGTCGCCA CCCATGGCTG AGAGCTC	ATHG
ATHR	GAGCTCTCAGCCATGGGTGGCGACAGC	
NTAL	GCTGTCAACA CCGCTGGCTG AGAGCTC	NTAG
NTAR	GAGCTCTCAGCCAGCGGTGTTGACAGC	
QTHL	GCTGTCCAAA CCCATGGCTG AGAGCTC	QTHG
QTHR	GAGCTCTCAGCCATGGGTTTGGACAGC	
NTFL	GCTGTCAACA CCTTTGGCTG AGAGCTC	NTFG
NTFR	GAGCTCTCAGCCAAAGGTGTTGACAGC	

trol. The Δ^6 fatty acid desaturase activity was absent in yeast cells transformed with the empty vector pYES2 (data not shown).

The Δ^6 fatty acid desaturase activity of RnD6C and the mutants in yeast was determined using linoleic acid (LA, $18{:}2^{\Delta 9,12}$) as the substrate. Their conversion rates are shown in Fig. 1C. For comparison, the mean desaturation activity for RnD6C is defined as 100%. The desaturation activities of other mutants are defined as RnD6C%. Values are the means obtained from at least three independent assays ± SD. RnD6C381 cannot use the D6D substrate LA (Fig. 1C), and RnD6CA381 had only a 29.4 ± 0.6% desaturase activity of RnD6C when LA was used as the substrate (Fig. 1C).

The desaturation rate and the conversion rate of enzymes tested by GC–MS are shown in Fig. 1C. The results indicate that the enzyme catalytic activity of K389T and Y447H was similar to that of RnD6C, and the enzyme catalytic activity of V394A, K395I, F411L, S436P, VK3945AI and IS4356VP were significantly decreased anywhere from 36.8 ± 3.01% to 80.1 ± 9.3% of RnD6C (Fig. 1C). Among the mutants, VK3945AI had the greatest decline (with only 36.8 ± 3.01% of catalytic activity of the control). However, I417T significantly improved the desaturase activity to 132.6 ± 15.6% of RnD6C.

3.3. The role of the last four amino acid residues

To test whether the last four amino acid residues play a role in catalysis, a series of mutant genes were constructed. In RnD6C, the amino acid residue N was mutated to H, A or Q; T mutated to S or A; and Y mutated to H, F or A, and they were named as NTHG,

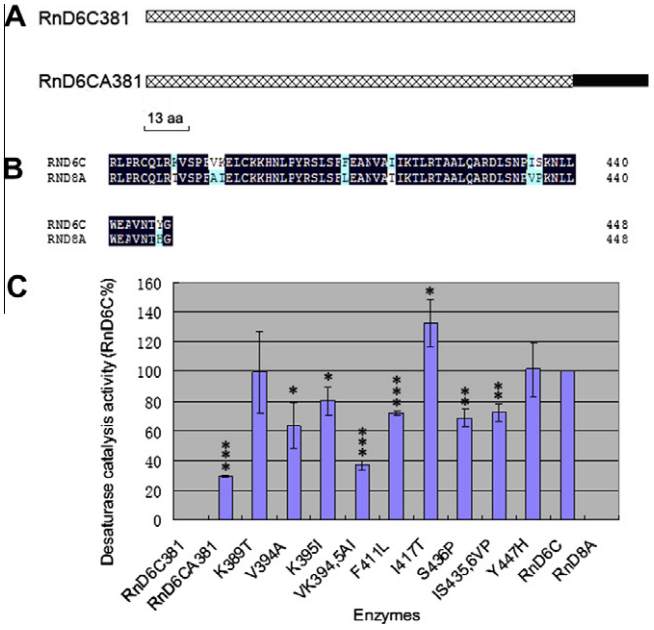

Fig. 1. Schematic structural diagrams and desaturation activities of the mutated proteins relative to RnD6C activity. (A) Schematic structural diagrams of RnD6C381 and RnD6CA381. Regions in ▨ originate from RnD6C, and regions in black are substituted from the corresponding region of RnD8A. (B) Schematic structural diagrams of RnD6C C-terminal mutation sites. (C) Desaturation activities of the mutated proteins relative to RnD6C activity. Conversion rates of mutated proteins in yeast were analyzed for the fatty acid LA following a 72 h induction. For comparison, the mean conversion rate of RnD6C was defined as 100%. Values are the mean of three independent assays ± SD. *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0005$.

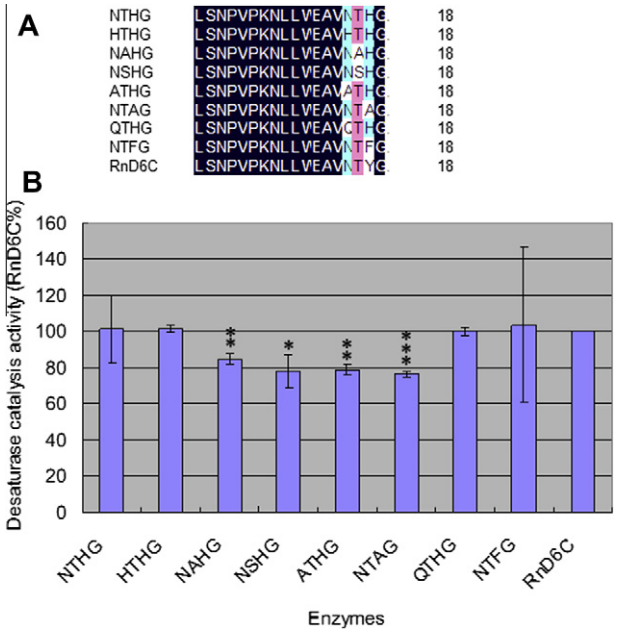

Fig. 2. Schematic structural diagrams of RnD6C C-terminal mutants and desaturation activities of the mutated proteins relative to RnD6C activity. (A) The mutants are named based on their corresponding amino acids (e.g., NTHG, NTAG and so on). (B) Desaturation activities of the mutant proteins relative to RnD6C activity. Conversion rates of the mutant proteins in yeast were analyzed for the fatty acid LA following a 72 h induction. For comparison, the mean conversion rate of RnD6C was defined as 100%. Values are the mean of three independent assays ± SD. *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0005$.

HTHG, NAHG, NSHG, ATHG, NTAG, QTHG, and NTFG (they were named using the last four amino acid residues) (Fig. 2A). The general principles of the mutagenesis are to mutate the chosen amino acids to an amino acid with similar chemical nature (such as T to S) or to alter the chemical nature of the residues (such as charged hydrophobic Y to non-charged hydrophilic A).

The D6D activity of RnD6C and the mutants in yeast was determined using LA as the substrate. The conversion rates of the wild-type and the mutants are shown in Fig. 2B. For comparison, mean desaturation activity for RnD6C is defined as 100%. The desaturation activities of the mutants are defined as RnD6C%. Values are the means obtained from at least three independent assays ± SD. We found that the conversion rates of mutants NTFG, HTHG, NTHG and QTHG were similar to that of RnD6C, and the conversion rates of mutants NAHG, ATHG, NSHG and NTAG were significantly decreased by approximately 20% (Fig. 2B).

4. Discussion

There is a lack of literature on the three dimensional structure of membrane-bound desaturases. The N-terminal of the D6D and the three conserved His boxes has been investigated in detail in several studies [8,9,11,12]. Only one study that focused on mutations of D8D Boof D8 and D6D Boof D6 mentioned the importance of C-terminal of D6D [4], but it did so without providing detailed data. We used blackcurrant D6D RnD6C and D8D RnD8A, which share high identity [16] but have different functions, to construct a series of chimeric genes and site mutant genes to study the relationship of functional region and enzyme activity of D6D C-terminus (Fig. 1A and B).

By analyzing the desaturase catalysis results, we found that RnD6C381 cannot use the D6D substrate LA and that the enzymatic activity of RnD6CA381 was decreased to 29.4 ± 0.6% of RnD6C when using LA as the substrate. These results indicated that the region of the C-terminus 67 aa of blackcurrant D6D was essential for the enzyme activity and might not be the substrate recognition region but a region related to the catalytic activity. There are only 8 different residues of RnD8A and RnD6C in the C-terminal 67 aa region, which may be the important site for D6D catalytic activity. We replaced these 8 different amino acid residues in RnD6C with corresponding sites of RnD8D and found that the enzyme activity of V394A, K395I, F411L, S436P, VK3945AI and IS4356VP was significantly decreased by up to approximately 60%; however, I417T improved the desaturase activity by more than 30% compared with that of RnD6C. Our results suggested that 7 of the 8 different amino acid residues between RnD6C and RnD8A are important for the D6D activity. In addition, mutating I to T at the 417th aa position in order to modify D6D to have a higher desaturase activity could be useful for producing GLA in crops in the future.

By comparing the published peptide sequences of D6D in plants, we found that the last four aa in the C-terminus are relatively conserved and almost all D6D contain His at the 447th aa site. However, of the sequences we cloned from blackcurrant, two had Tyr at the 447th site [16]. We analyzed the functions of the last four aa by mutagenesis (Fig. 2A and B). The results demonstrated that changing the 447th residue T to H or F in the RnD6c did not change its enzymatic activity. However, except for mutants NTFG, HTHG, NTHG and QTHG, which were similar to RnD6C in enzymatic activity, NAHG, ATHG, NSHG and NTAG had significantly lower enzymatic activity, a decrease by approximately 20% (Fig. 2B). Thus, aa residues N, T, Y (H) play a certain role in the enzyme activity of D6D.

In conclusion, we found several interesting results: (1) the C-terminus of blackcurrant D6D, consisting of 67 aa, was essential for its enzymatic activity; (2) this region might not be the substrate recognition region but is related to the catalytic activity; (3) seven of eight different amino acid residues between RnD6C and RnD8A

in the C-terminus 67 aa region have an important role for the D6D activity; (4) mutation of I to T at the 417th aa significantly improved the D6D enzymatic activity; and (5) N, T, and Y(H) in the last 4 aa in C-terminus also play a certain role in D6D enzyme activity.

Acknowledgments

This research was supported by a project (2011ZX08009-003-004) from the Ministry of Agriculture of China for transgenic research and a project (2011CB200902) from the Ministry of Science and Technology of China.

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