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ORIGINAL ARTICLE

Four Amino Acid Residues Influence the Substrate Chain-Length and Regioselectivity of *Siganus canaliculatus* Δ4 and Δ5/6 Desaturases

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Abstract Although ω3- and ω6- desaturases have been well studied in terms of substrate preference and regiospecificity, relatively little is known about the membrane-bound, "front-end" long chain fatty acid desaturases, such as Δ4, Δ5 or Δ6 desaturases. The first vertebrate Δ4 desaturase was recently identified in the marine teleost fish *Siganus canaliculatus* (*S. canaliculatus*), which also possesses a bifunctional Δ5/6 desaturase. These two long chain polyunsaturated fatty acid desaturases are very different in terms of regiospecificity and substrate chain-length, but share an unusually high degree of amino acid identity (83 %). We took advantage of this similarity by constructing a series of chimeric enzymes, replacing regions of one enzyme with the corresponding sequence of the other. Heterologous expression of the chimeric series of enzymes in yeast indicated that the substitution of a four amino acid region was sufficient to convert a Δ4 desaturase to an enzyme with Δ6 desaturase activity, and convert a Δ5/6 desaturase to an enzyme with a low level of Δ4 desaturase activity. In addition, enzymes having both Δ4 and Δ6 desaturase activities were produced by single or double amino acid substitutions within this four-amino acid region.

Keywords Desaturase · Substrate specificity · Chain-length · Regioselectivity · *Siganus canaliculatus*

Abbreviations

ALA	α-Linolenic acid, 18:3n-3
ARA	Arachidonic acid, 20:4n-6
DPA	Docosapentaenoic acid, 22:5n-3
DHA	Docosahexaenoic acid, 22:6n-3
EPA	Eicosapentaenoic acid, 20:5n-3
GLA	γ-Linolenic acid, 18:3n-6
DGLA	Dihomo-γ-linolenic acid, 20:3n-6
SDA	Stearidonic acid, 18:4n-3
VLCPUFA	Very long chain polyunsaturated fatty acid
S. canaliculatus	*Siganus canaliculatus*
S. cerevisiae	*Saccharomyces cerevisiae*

Introduction

Fatty acid desaturases are enzymes that are involved in the biosynthesis of unsaturated fatty acids. These enzymes act by removing two hydrogen atoms from the fatty acid hydrocarbon chain, resulting in the formation of a double bond [1, 2]. "Front-end" desaturases characteristically contain a cytochrome b_5-like domain at the N-terminus of the protein, and introduce a double bond between the carboxyl-end of the fatty acid and a pre-existing double bond. The cytochrome b_5 domain plays a role as an electron donor during desaturation, and a heme-binding "HPGG" motif is present in the cytochrome b_5 domain of almost all desaturases [3–5]. Previous studies showed that the cytochrome b_5 domain is essential for the function of

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desaturases because point mutations that altered the histidine residues resulted in a loss of desaturase activity [6]. Yeast Δ9 desaturase is different than 'front-end' desaturases in that it has a C-terminal cytochrome b_5 domain, which was also shown to be functionally important [7]. The regioselectivity of a desaturase is demonstrated by the selective insertion of a double bond at a specific position in the fatty acyl chain. "Front-end" desaturases generally have high regioselectivity and substrate specificity due to their structure [2].

Three conserved histidine boxes, generally containing the consensus sequences H–X (3-4) –H, H–X (2-3) –H–H and H–X (2-3) –H–H, are typically found in membrane-bound fatty acid desaturases [8, 9]. These three histidine boxes form the active site of desaturases, and are involved in the formation of a diiron complex [1, 3]. By using site-directed mutagenesis, Shanklin and colleagues showed that each of the eight histidine residues in these boxes is essential for the catalytic function of the stearoyl-CoA Δ9 desaturase from rat liver [10]. In addition, in 1998, Broun et al. [11] modified amino acids in close proximity to the histidine boxes and were able to convert an oleate 12-desaturase to a hydroxylase and vice versa. Similarly, amino acid modifications close to the histidine boxes of *Claviceps purpurea* Δ12 and Δ15 desaturases affected the catalytic properties and regioselectivity of the enzymes [12]. In "front-end" desaturases, the first histidine residue in the third histidine box is usually replaced by glutamine. This glutamine residue appears to be essential for enzyme activity, since substituting histidine in this position in a borage Δ6 desaturase abolished enzymatic activity [13]. Furthermore, for the borage front-end Δ6-fatty acid and Δ8-sphingolipid desaturases, the transmembrane domains and carboxyl termini were shown to play a major role in determining substrate specificity and regioselectivity, possibly by contributing to the formation of the substrate binding site [14].

Several pathways have been suggested for the formation of very long chain Δ4-desaturated fatty acids such as docosahexaenoic acid (DHA, 22:6n-3). One route for DHA synthesis uses 18:3n-3 as a substrate, which undergoes a Δ6 desaturation and a Δ6 elongation to produce 20:4n-3 followed by a Δ5 desaturation step and a Δ5 elongation to form 22:5n-3 [15, 16]. Conventionally, due to the lack of observed Δ4 activity in mammals [17], the main route for production of 22:6n-3 from 22:5n-3 was thought to proceed via a Δ7 elongation and a Δ6 desaturation, producing 24:6n-3, followed by a β-oxidation reaction to remove two carbon units from the fatty acid [18, 19]. A simpler route for the production of 22:6n-3 from 22:5n-3 is via Δ4-desaturation, and it is noteworthy that the first Δ4 desaturase was cloned in 2001 from the marine protist *Thraustochytrium* [20]. Since then, several other groups have identified Δ4 desaturases from protists and microalgae [21–27]. Recently, the first vertebrate Δ4 desaturase gene was isolated from *S. canaliculatus* [28], which suggests there might be a more straightforward mechanism available for DHA biosynthesis in vertebrates as well.

To date, the only fish species with demonstrated Δ5 and Δ6 fatty acid desaturation activity are Atlantic salmon, zebrafish and *S. canaliculatus* [28–31]. Atlantic salmon have separate genes encoding Δ5 and Δ6 desaturation activities, whereas zebrafish and *S. canaliculatus* possess bifunctional Δ5/6 fatty acid desaturase genes. *S. canaliculatus* Δ4 and Δ5/6 desaturases are active with both ω3 and ω6 substrates; the Δ4 desaturase acts on 22:5n-3 and 22:4n-6 and the Δ5/6 desaturase acts on 18:3n-3, 18:2n-6, 20:4n-3, and 20:3n-6 [28]. However, both enzymes were shown to have a preference for ω3 fatty acids in the yeast expression system [28]. Although *S. canaliculatus* Δ4 and Δ5/6 are functionally different in terms of specificity for substrate chain-length and regioselectivity, these enzymes share a high degree of amino acid identity (83 %). We initiated this study in an attempt to determine which regions or residues of the proteins encoded by these genes caused the functional divergence between these two highly similar desaturases. To identify the amino acid residues that determine the differences in substrate specificity and regioselectivity, we designed a series of constructs containing substitutions between the Δ4 and Δ5/6 desaturases of *S. canaliculatus*. We then analyzed the effects of these substitutions on the substrate specificity and catalytic activity of the enzymes by functional characterization in yeast.

Materials and Methods

Materials

Growth and induction media for yeast expression were purchased from MP Biologicals (Solon, OH). Fatty acid (DPA and ALA) substrates were purchased from Nu-Chek Prep Inc (Elysian, MN). Phusion high fidelity DNA polymerase was obtained from New England Biolabs (Ipswich, MA), and Taq polymerase from Invitrogen (Burlington, ON). All HPLC grade solvents were purchased from EMD Inc. (Mississauga, ON) and Fisher Scientific (Ottawa, ON). All chemicals if not mentioned otherwise, were purchased from Sigma-Aldrich (Oakville, ON).

S. canaliculatus Δ4 and Δ5/6 Desaturases

Sequences encoding the *S. canaliculatus* Δ4 (GenBank accession number: GU594278) and Δ5/6 (GenBank accession number: EF424276) desaturases were synthesized by the Life Technologies Corporation (Burlington, ON) based

on the GenBank database sequences. Synthetic genes were cloned into pYES2.1/V5-His-TOPO (Invitrogen) and sequenced prior to being used in further experiments.

Construction of Vectors

Chimeric enzymes were constructed from Δ4 and Δ5/6 fatty acid desaturases by overlap extension PCR ('sewing' PCR). Desired consecutive fragments were amplified from individual genes or constructs using primers that included homologous overlapping regions to produce sequential DNA fragments with overlapping 3′ and 5′ termini. These fragments were isolated and 'sewn' together by a second PCR to form a chimeric gene. The synthesized *S. canaliculatus* Δ4 and Δ5/6 desaturases served as templates for initial chimeric constructs, and for subsequent chimeric genes, either the *S. canaliculatus* desaturases or previously constructed chimeric genes were used as templates. PCR was performed using Phusion high fidelity DNA polymerase. After the final overlap extension PCR giving the full-length product, the amplified DNA fragment was cloned into the pYES2.1/V5-His-TOPO vector according to the manufacturer's instructions, and the plasmid DNA was isolated using an EZ-10 spin column plasmid DNA miniprep kit (Bio Basic Inc.). After sequencing, plasmids were transformed into the *Saccharomyces cerevisiae* (*S. cerevisiae*) yeast strain INVSc1 (Invitrogen) using the EasyComp transformation kit (Invitrogen). All primers used in these experiments are listed in Supplementary Table 1.

Expression in Yeast

For functional expression of the desaturases, yeast cultures were grown overnight at 30 °C in drop out base (DOB-URA: 1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, and complete supplement mixture minus uracil) containing 2 % glucose. The OD_{600} of the overnight yeast cultures were measured and standardized between samples. The samples were washed with DOB-URA containing 2 % galactose and expression was carried out for 3 days at 20 °C in the same media supplemented with fatty acids (250 μM DPA or ALA) and 0.01 % tergitol (NP-40).

Fatty Acid Analysis

After 3 days of growth in the presence of exogenously supplied fatty acids, the yeast cells were collected by centrifugation at 1,500×*g* for 3 min and washed once with induction buffer (2 % galactose) then washed again with sterile distilled water. 2 mL of 3N-methanolic HCl was added to each sample and the samples were heated at 80 °C for 40 min, then cooled to room temperature, and the hexane phase containing the fatty acid methyl esters was partitioned from the aqueous phase by the addition of 1 mL 0.9 % NaCl and 2 mL hexane, followed by centrifugation. The hexane phase was transferred to glass vials and air-dried under a gentle stream of liquid nitrogen gas and was resuspended in 100 μL of hexane before being analyzed by gas chromatography, using an Agilent 6890N gas chromatograph equipped with a DB-23 column (30 m × 0.25 mm) with 0.25 μm film thickness (J&W Scientific). The column temperature was maintained at 160 °C for 1 min, then raised to 240 °C at a rate of 4 °C/min. The desaturation efficiency (%) was calculated as: product/(substrate + product) × 100.

Results

S. canaliculatus Δ4 and Δ5/6 Desaturases Have Similar Sequences and Structures

The deduced protein sequences of the Δ4 and Δ5/6 desaturase genes of *S. canaliculatus* have 445 and 443 amino acids, respectively, with the two amino acid gap located in the N-terminal region of the alignment (Fig. 1). Based on membrane topology analysis (TOPCONS [32]; restraining N- and C-terminal inside cytoplasmic area; Fig. 2), both the *S. canaliculatus* Δ4 and Δ5/6 desaturases have four predicted transmembrane domains. Both desaturases have three conserved histidine boxes, and an N-terminal cytochrome b_5-like domain, which are the characteristic features of a front-end microsomal desaturase [28]. Due to the high similarity in amino acid sequence between the two desaturases, we were able to rationally design primers to produce chimeras based on aligning the *S. canaliculatus* cDNAs and predicted amino acid sequences (Fig. 1). Specific corresponding regions were selected and exchanged between the two desaturases to form chimeras. Regions that were quite highly conserved, as well as less conserved regions were substituted.

Supplementary Table 1 lists the primers used for constructing the chimeras and for performing site-directed mutagenesis to identify the roles of specific residues. This set of primers allowed us to perform substitutions of domains distributed in the hydrophobic transmembrane domains, C-terminal and N-terminal regions and linkers between transmembrane domains, based on the topology analysis shown in Fig. 2.

Functional Characterization of Chimeric *S. canaliculatus* Desaturases Identifies a Four Amino Acid Region Influencing Substrate Specificity

Both *S. canaliculatus* desaturases show a preference for ω3 fatty acids [28]. Therefore, the ω3 fatty acids DPA and

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  Sc D4     (1) MGGGGQLGESGENGCKSAAGVYTWEEVQHHSNRNDQWLVIDRKVYNVTQWAKRHPGGFRV
Sc D5/6     (1) MGGGGQPRESGEP--GSSPAVYTWEEVQHHSSRNDQWLVIDRKVYNISQWAKRHPGGYRV

  Sc D4    (61) LNHYAGEDATEAFTAFHPDIKFVQKYMKPLLVGELAATEPSQDQDKNAALIQDFHTLRQQ
Sc D5/6    (59) IGHYAGEDATEAFTAFHPDLKFVQKFLKPLLIGELAATEPSQDRNKNAALIQDFHTLRQQ

  Sc D4   (121) AESEGLFQARPLFFLLHLGHILLLEALALLMVWHWGTGWLQTLLCAVMLATAQSQAGWLQ
Sc D5/6   (119) AESEGLFQARPLFFLLHLGHILLLEALALLMVWHWGTGWLQTLLCAVMLATAQSQAGWLQ

  Sc D4   (181) HDFGHLSVFKKSRWNHLVHKFVIGHLKGASANWWNHRHFQHHAKPNIFKKDPDINMVDLF
Sc D5/6   (179) HDFGHLSVFKKSRWNHLVHHFVIGHLKGASANWWNHRHFQHHAKPNIFKKDPDINMVDLF

                                                        5 & 6
  Sc D4   (241) VLGETQPVEYGIKKIKNMPYNHQHKYFFLVAPPLLIPVFYNYNIMMTMITRRDYVDLSWA
Sc D5/6   (239) VLGETQPVEYGVKKIKLMPYNHQHQYFHLIGPPLLIPVFFHYQLLKIMISHRYWLDLVWC
                                  3 & 4
  Sc D4   (301) MTFYIRYMLCYVPVYGLFGSLALMMFARFLESHWFVWVTQMSHLPMDIDNDKRRDWLSMQ
Sc D5/6   (299) LSFYLRYMCCYVPVYGLFGSVVLIVFTRFLESHWFVWVTQMNHLPMDINYENHNDWLSMQ
                                                    1 & 2
  Sc D4   (361) LQATCNIEKSFFNDWFSGHLNFQIEHHLFPRMPRHNYHLVAPQVQTLCEKHGIPYEVKTL
Sc D5/6   (359) LQATCNVEQSLFNDWFSGHLNFQIEHHLFPTMPRHNYHLVVPRVRALCEKHEIPYQVKTL

  Sc D4   (421) WKGMVDVVRALKKSGDLWLDAYLHK
Sc D5/6   (419) PQAFADIIRSLKNSGELWLDAYLHK
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Fig. 1 Alignment of deduced amino acid sequences of *S. canaliculatus* Δ4 desaturase (GenBank accession number: GU594278) and Δ5/6 desaturase (GenBank accession number: EF424276) generated by VectorNTI software. Non-identical residues are highlighted in *grey*. The cytochrome b_5-like domain is indicated by a *wavy line*. The four amino acid residues that determine the functional divergence between the two genes are shown in *bold*. Histidine boxes are *underlined* by *dotted lines* and the putative transmembrane domains are *underlined* with *solid lines*. To provide reference points for chimeric constructs, the starting positions of the major substitutions in chimeras *1–6* are identified by *arrows*

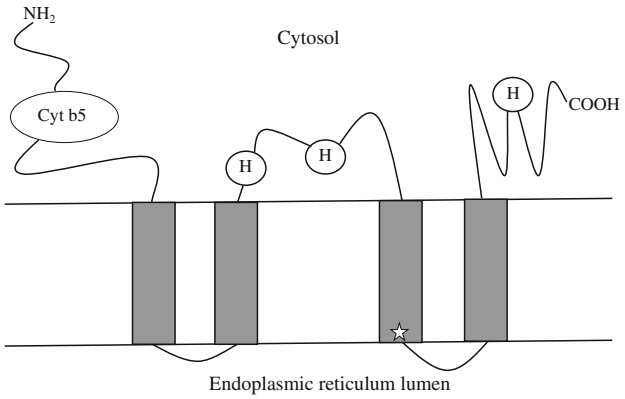


Fig. 2 Putative protein topology of *S. canaliculatus* Δ4 and Δ5/6 desaturases based on TOPCONS membrane topology prediction software. The membrane topology was generated by restraining N- and C-terminal regions to the inside (cytoplasmic) area. H denotes *histidine box*. *Star* indicates the approximate location of the four amino acids residues involved in substrate chain-length specificity and regiospecificity

ALA, which are substrates for the native Δ4 and Δ5/6 desaturases, respectively, were used throughout this study. As a negative control, *S. cerevisiae* INVSc1 cells were transformed with the empty pYES2.1/V5-His-TOPO vector. Desaturation of exogenously supplied ALA or DPA was not observed in the negative control (Fig. 3). Expression of wild-type *S. canaliculatus* Δ4 and Δ5/6 desaturases in yeast showed desaturase activities of 12.0 ± 0.5 % (DPA) and 22.7 ± 2.5 % (ALA), respectively, as demonstrated by the representative chromatograms in Fig. 4a, b. Supplementary Table 2 gives the levels of substrate and product as a percentage of total yeast fatty acids for the constructs shown in Fig. 4. The Δ4 desaturase did not show activity with ALA, nor did the Δ5/6 desaturase show activity with DPA (Fig. 4a, b).

When the *S. canaliculatus* Δ4 desaturase sequence from the amino acid sequence MPRH onward (amino acid residues 392–445), representing approximately the last one-tenth of the enzyme, was replaced with the Δ5/6 sequence, the enzyme maintained the same substrate specificity but the conversion level dropped by approximately 43 % (Sig-1C, Fig. 5a). The reverse construct (Sig-2C, Fig. 5a) maintained the substrate specificity of the Δ5/6 enzyme but fatty acid conversion was reduced by approximately 30 %. Since the last tenth of the enzymes do not appear to contribute to substrate specificity, chimeras with domain swapping from the PVYG amino acid location onward, representing approximately the last third of the enzyme (Δ4 desaturase amino acid residues 313–445), were constructed (Sig-3C and 4C; Figs. 4c, d, 5a). No change in substrate specificities were observed between the two chimeras but the enzyme activities of Sig-3C and 4C were reduced by

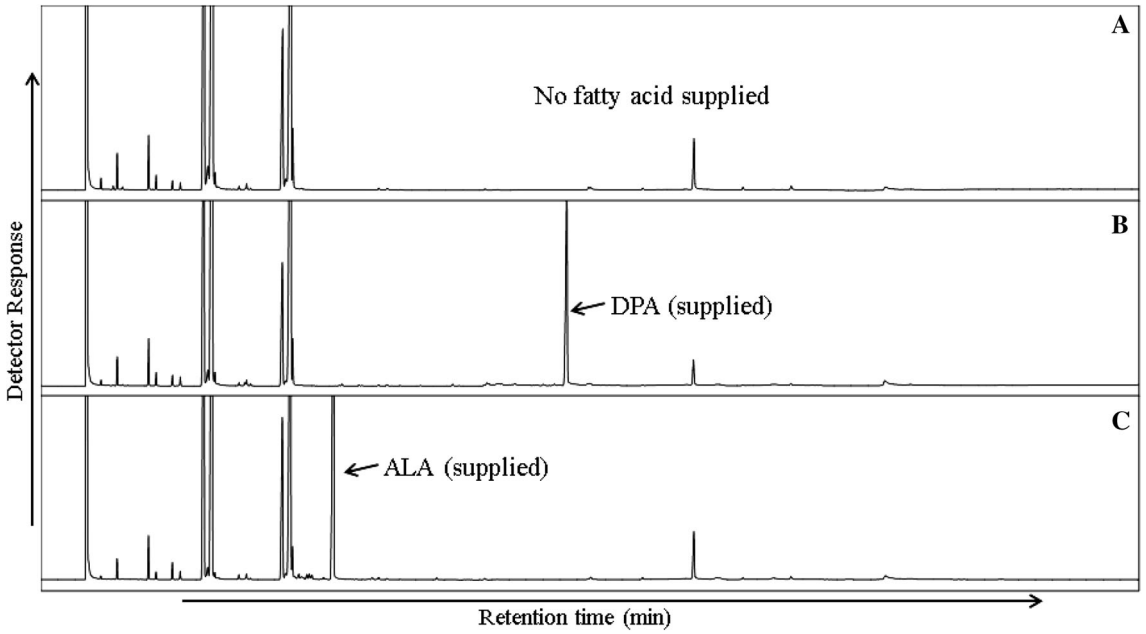


Fig. 3 Gas chromatograms of fatty acid methyl esters derived from yeast cells containing an empty pYES2.1/V5-His-TOPO vector. **a** No fatty acid supplied; **b** DPA supplied exogenously; **c** ALA supplied exogenously

approximately 30 and 45 %, respectively, compared to wild-type (Fig. 5a).

The regions from LIPV onward, representing approximately the last 40 % of the coding regions, were exchanged in the corresponding chimeras Sig-5C and Sig-6C (Fig. 5a). The switch from Δ4 to Δ5/6 sequence at LIPV caused Sig-5C to lose Δ4 activity but gain Δ6 activity, showing a conversion level of approximately 23 % with ALA (Sig-5C; Figs. 4e, 5a). Conversely, the reverse construct lost Δ6 activity but gained activity with DPA (Sig-6C; Figs. 4f, 5a). Thus, the region responsible for substrate specificity appeared to be located between the LIPV and PVYG regions (amino acid residues: Δ4, 275–316; Δ5/6, 273–314) of these enzymes, as shown in Fig. 1. Because the Δ5/6 desaturase is considered to be a bifunctional enzyme, a Δ5 desaturase substrate (dihomo-γ-linolenic acid, DGLA) was also tested. However, the wild type *S. canaliculatus* Δ5/6 desaturase showed a very low conversion percentage with DGLA (1.63 ± 0.23 %). Sig-5C also showed a low level of Δ5 desaturation (1.31 ± 0.05 %); this coincided with the switch from Δ4 desaturation to Δ6 desaturation observed with Sig-5C. Although other constructs were also tested with DGLA, activity was not detectable, likely due to the very low Δ5 activity of the native protein. Thus the Δ5 substrate did not prove useful in pinpointing functional regions of enzymes in these experiments.

To confirm the residues between LIPV to PVYG alone were responsible for substrate specificity with ALA and DPA, a pair of chimeric genes (Sig-7C and 8C; Fig. 5a) with substitutions of only this region were constructed by PCR. The gene containing Δ5/6 desaturase sequence in this area and Δ4 sequence throughout the rest of the construct desaturated only ALA (11.5 ± 1.2 %) while the reverse construct had activity only with DPA (3.8 ± 0.2 %), demonstrating that the region between amino acids LIPV and PVYG controls substrate specificity. This area includes part of the third and fourth predicted transmembrane domains along with the linker region (Figs. 1, 2).

To further dissect this region, chimeric genes were constructed that divided the region between LIPV and PVYG of the *S. canaliculatus* Δ4 and Δ5/6 desaturases into three fractions (end of linker to fourth transmembrane domain: WAMT/WCLS to PVYG, linker region between third and fourth transmembrane domain: TMI/IMI to WAMT/WCLS and third transmembrane domain to start of linker region: LIPVF to TMI/IMI). When expressed in yeast cells, Sig-9C, which is an *S. canaliculatus* Δ4 desaturase gene with a substitution of the region encoding the amino acids from WCLS to PVYG from the Δ5/6 desaturase sequence (Fig. 5a) converted 4.0 ± 0.5 % of DPA to DHA, while the reverse construct (Sig-10C; Fig. 5a) converted ALA to SDA at a level of 14.9 ± 1.4 %, indicating that the fourth transmembrane domain is not relevant in substrate specificity but has an effect on the catalytic activities of the enzymes. Moreover, Sig-11C and its reverse gene construct (Sig-12C; Fig. 5a) which have sequence exchanges between the linker region of third and fourth transmembrane domains (sequence from TMI/IMI to WAMT/WCLS), also retained their original substrate specificity although enzyme activity was reduced to about half that of the respective wild-type

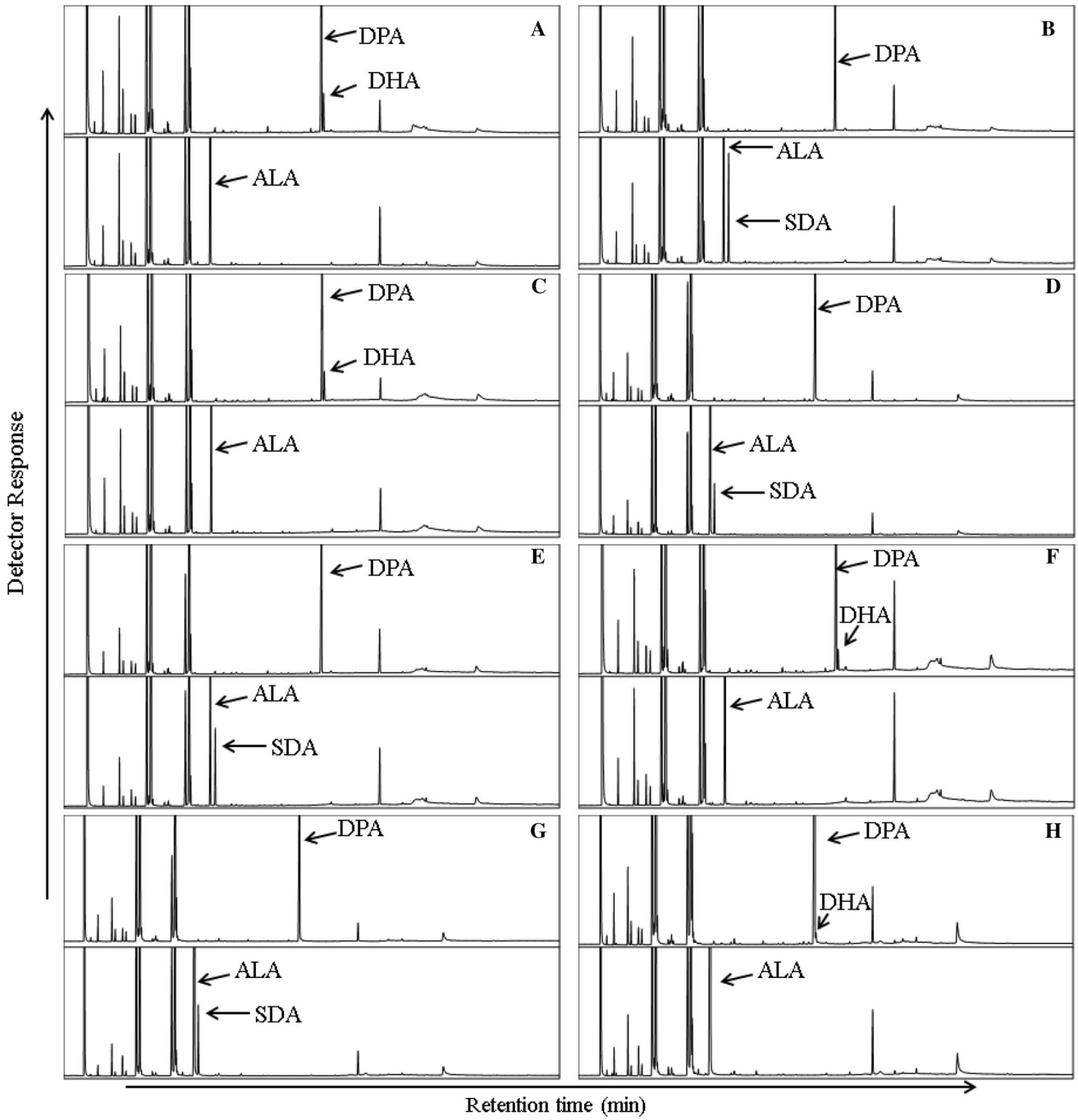


Fig. 4 Gas chromatograms of fatty acid methyl esters derived from yeast cells expressing *S. canaliculatus* wild-type and mutated desaturases. **a** *S. canaliculatus* Δ4 desaturase; **b** *S. canaliculatus* Δ5/6 desaturase; **c** chimeric *S. canaliculatus* desaturase Sig-3C; **d** chimeric *S. canaliculatus* desaturase Sig-4C; **e** chimeric *S. canaliculatus* desaturase Sig-5C; **f** chimeric *S. canaliculatus* desaturase Sig-6C; **g** chimeric *S. canaliculatus* desaturase Sig-17C; **h** chimeric *S. canaliculatus* desaturase Sig-18C. *Top* and *bottom* panels show results from cultures supplied with DPA and ALA, respectively

genes (Fig. 5a). These data suggest that the linker region between the third and fourth transmembrane domain (TMI/IMI to WAMT/WCLS) on the *S. canaliculatus* Δ4 and Δ5/6 desaturases is not the critical region for substrate specificity, although sequence switching in these regions greatly reduces catalytic activities. However, when the regions between the third transmembrane domain to the start of linker region, involving the sequence from LIPV to IMI/TMI (Sig-13C and 14C; Fig. 5a) were substituted, there was a shift in substrate specificity. The chimeric *S. canaliculatus* Δ4 enzyme (Sig-13C; Fig. 5a) desaturated only ALA (13.1 ± 1.8 %) but no longer desaturated DPA, therefore

this substitution allowed the gene to gain Δ5/6 activity but completely abolished the Δ4 activity. In contrast, the reverse construct (Sig-14C; Fig. 5a) gained Δ4 activity as demonstrated by DPA desaturation (3.4 ± 0.2 %) but completely lost its original Δ5/6 activity. Thus, a region that appears to be within the third transmembrane domain critically affects substrate specificity.

To examine whether the 8 amino acids (*S. canaliculatus* Δ4 desaturase: LIPVFYNYNIMMTMI; *S. canaliculatus* Δ5/6 desaturase: LIPVFFHYQLLKIMI) between the LIPVF to TMI/IMI region were all required for substrate specificity or if only specific amino acids were critical, we made constructs with alterations in this region. To narrow down the possibilities, we divided the 8 amino acids into 2 portions. We constructed an *S. canaliculatus* Δ4 desaturase gene (Sig-15C; Fig. 5b) in which the IMMT amino acid sequence was altered to LLKI to resemble the *S. canaliculatus* Δ5/6 desaturase at the same location. This did not affect the substrate specificity of the Δ4 desaturase gene, as it desaturated DPA (2.9 ± 0.3 %) to generate DHA and had no activity on ALA. Similarly, the reverse gene construct with alteration of the *S. canaliculatus* Δ5/6 desaturase (Sig-16C; Fig. 5b) from LLKI to IMMT (corresponding residues in *S. canaliculatus* desaturase Δ4) did not affect substrate specificity (16.3 ± 1.1 %, ALA). However, substitution of the Δ4 desaturase amino acid sequence YNYN to the Δ5/6 desaturase sequence FHYQ at the same location (Sig-17C; Figs. 4g, 5b) changed the substrate specificity from DPA to ALA (12.5 ± 0.8 %; Fig. 4g). Likewise, altering the *S. canaliculatus* Δ5/6 desaturase sequence from FHYQ to the corresponding YNYN sequence (Sig-18C; Figs. 4h, 5b) resulted in a lost of Δ5/6 desaturase activity but allowed a low level of Δ4 desaturase activity (0.5 ± 0.1 %; Fig. 4h). Thus, the four amino acids YNYN in the *S. canaliculatus* Δ4 desaturase and the corresponding amino acids FHYQ in the Δ5/6 desaturase play a role in regulating substrate specificity and/or enzyme activity.

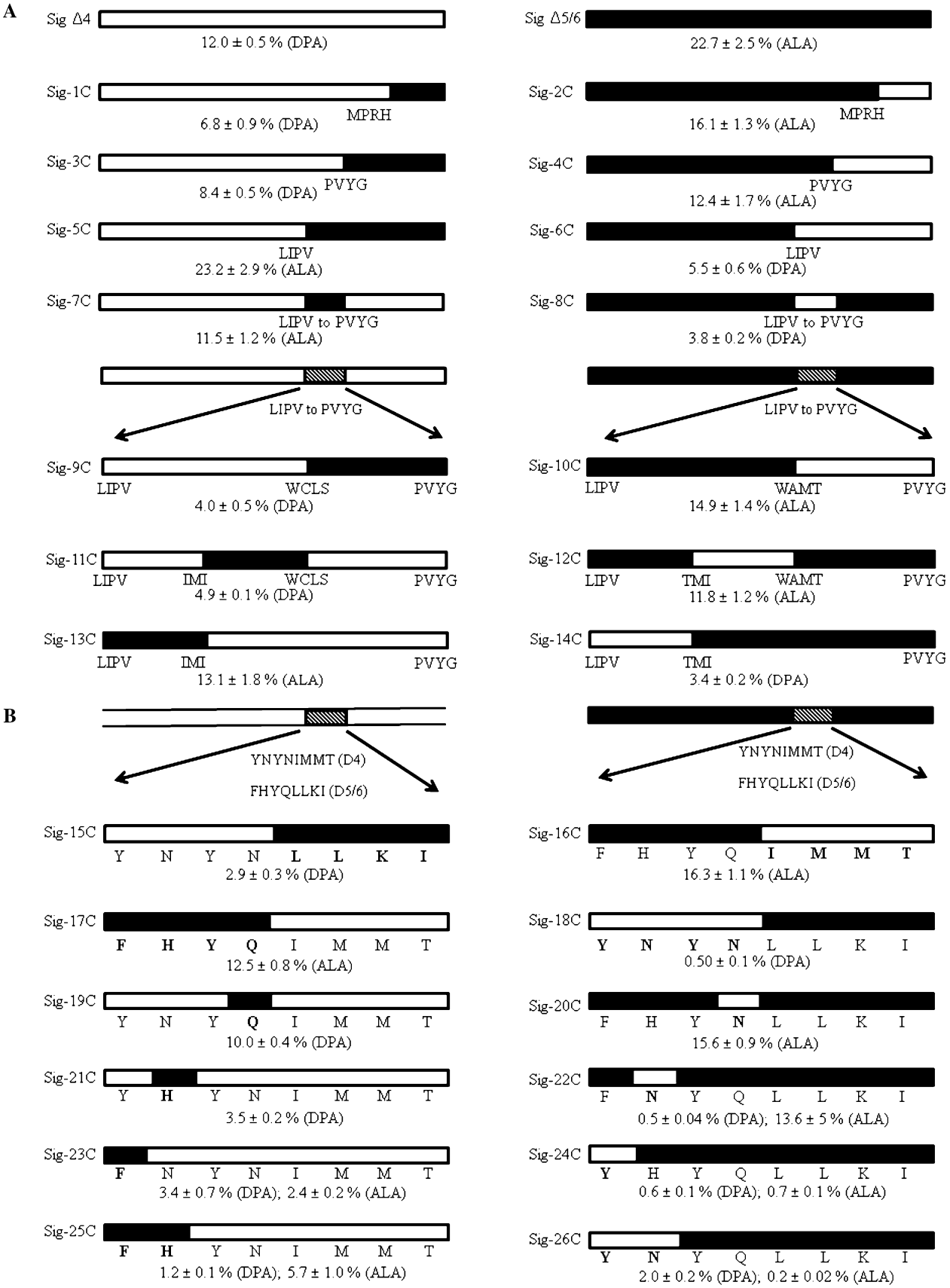
Substitution of Amino Acids within the Four Amino Acid Region Produces Enzymes with Both Δ4- and Δ6-Desaturase Activities

After indentifying a four amino acid region that influenced substrate specificity, substitution of individual amino acids within this region was performed (Table 1). An amino acid substitution at position 4 (Table 1) of the critical region showed no effect on substrate specificity in either the Δ4 (Sig-19C; Fig. 5b) or Δ5/6 desaturases (Sig-20C; Fig. 5b), however, decreases in substrate conversion levels (10.0 ± 0.4 %, DPA for Sig-19C and 15.6 ± 0.9 %, ALA for Sig-20C; Fig. 5b) were observed. When a single amino acid was substituted at position 2 (N to H or vice versa), the substrate specificity of the point-mutated Δ4 desaturase (Sig-21C; Fig. 5b) remained the same (DPA) although enzyme activity was reduced to 3.5 ± 0.2 %, which is about a 71 % reduction in activity compared to wild-type. The Δ5/6 desaturases with an amino acid substitution at position 2 in the critical region (Sig-22C; Fig. 5b) not only desaturated ALA (13.6 ± 5.0 %) but also produced DHA at a low level (0.5 ± 0.04 %). Interestingly, the Sig-23C (Δ4 desaturase; Fig. 5b) and Sig-24C (Δ5/6 desaturase; Fig. 5b) with amino acid substitutions in the corresponding residues at position 1 of the critical region were able to desaturate both DPA and ALA at low levels. Since positions 1 and 2 seem to play a more important role in the substrate specificity, we rationally engineered desaturases with two amino acid substitutions in this region (Sig-25C and Sig-26C; Fig. 5b). Site-directed substitution of both of these amino acids concurrently also generates enzymes with non-specific desaturation (Sig-25 and Sig-26, Fig. 5b), although a stronger efficiency toward the preferred substrate of the enzyme from which these two amino acids originated was observed. However, mutations including the amino acids at position 1, 2 and 4 (Sig-17C and 18C; Fig. 5b) restored specificity, even though single mutations at the fourth position did not directly affect substrate specificity (Sig-19C and -20C, Fig. 5b). This suggests some type of interaction between these three amino acids affects the substrate specificity of the enzymes studied here.

Discussion

Front-end desaturases such as the *S. canaliculatus* Δ4 and Δ5/6 desaturases are involved in the biosynthetic pathway of very long chain polyunsaturated fatty acids (VLCPUFA) including DHA, arachidonic acid (ARA), eicosapentaenoic acid (EPA), γ-linolenic acid (GLA) and SDA. These VLCPUFA have profound effects on the structural function of membranes, and also have important health effects in humans [25, 33–36], making them important products for nutraceutical and pharmaceutical industries.

A number of Δ4 and Δ6 desaturases from non-vertebrate sources have previously been identified, including enzymes from *Thraustochytrium* sp., *Pavlova lutheri*, *Thalassiosira pseudonana* and *Euglena gracilis* [20–22, 37]. However, the amino acid similarity among these front-end desaturases tends to be low. For example, *Thraustochytrium* sp. Δ4 (GenBank accession number: AF489589) and Δ5 (GenBank accession number: AF489588) desaturases share only 19 % identity, and *Mortierella alpina* Δ5 (GenBank accession number: AF054824) and Δ6 (GenBank accession number: AF110510) desaturases have only 23 % identity in amino acid sequence. The low sequence similarity among


A
Sig Δ4
12.0 ± 0.5 % (DPA)
Sig Δ5/6
22.7 ± 2.5 % (ALA)
Sig-1C
MPRH
6.8 ± 0.9 % (DPA)
Sig-2C
MPRH
16.1 ± 1.3 % (ALA)
Sig-3C
PVYG
8.4 ± 0.5 % (DPA)
Sig-4C
PVYG
12.4 ± 1.7 % (ALA)
Sig-5C
LIPV
23.2 ± 2.9 % (ALA)
Sig-6C
LIPV
5.5 ± 0.6 % (DPA)
Sig-7C
LIPV to PVYG
11.5 ± 1.2 % (ALA)
Sig-8C
LIPV to PVYG
3.8 ± 0.2 % (DPA)
LIPV to PVYG
LIPV to PVYG
Sig-9C
LIPV WCLS PVYG
4.0 ± 0.5 % (DPA)
Sig-10C
LIPV WAMT PVYG
14.9 ± 1.4 % (ALA)
Sig-11C
LIPV IMI WCLS PVYG
4.9 ± 0.1 % (DPA)
Sig-12C
LIPV TMI WAMT PVYG
11.8 ± 1.2 % (ALA)
Sig-13C
LIPV IMI PVYG
13.1 ± 1.8 % (ALA)
Sig-14C
LIPV TMI PVYG
3.4 ± 0.2 % (DPA)
B
YNYNIMMT (D4)
FHYQLLKI (D5/6)
YNYNIMMT (D4)
FHYQLLKI (D5/6)
Sig-15C
Y N Y N L L K I
2.9 ± 0.3 % (DPA)
Sig-16C
F H Y Q I M M T
16.3 ± 1.1 % (ALA)
Sig-17C
F H Y Q I M M T
12.5 ± 0.8 % (ALA)
Sig-18C
Y N Y N L L K I
0.50 ± 0.1 % (DPA)
Sig-19C
Y N Y Q I M M T
10.0 ± 0.4 % (DPA)
Sig-20C
F H Y N L L K I
15.6 ± 0.9 % (ALA)
Sig-21C
Y H Y N I M M T
3.5 ± 0.2 % (DPA)
Sig-22C
F N Y Q L L K I
0.5 ± 0.04 % (DPA); 13.6 ± 5 % (ALA)
Sig-23C
F N Y N I M M T
3.4 ± 0.7 % (DPA); 2.4 ± 0.2 % (ALA)
Sig-24C
Y H Y Q L L K I
0.6 ± 0.1 % (DPA); 0.7 ± 0.1 % (ALA)
Sig-25C
F H Y N I M M T
1.2 ± 0.1 % (DPA); 5.7 ± 1.0 % (ALA)
Sig-26C
Y N Y Q L L K I
2.0 ± 0.2 % (DPA); 0.2 ± 0.02 % (ALA)

◀**Fig. 5** Schematic representation of *S. canaliculatus* Δ4 and Δ5/6 desaturase chimera gene constructs with associated conversion percentages. **a** Substitution of domains within enzymes. Chimeric constructs Sig 9C, 11C, and 13C are *S. canaliculatus* Δ4 desaturase sequences with substitutions in the LIPV to PVYG region, whereas Sig 10C, 12C, and 14C are *S. canaliculatus* Δ5/6 sequences with substitutions in this region. **b** Substitutions within a critical eight amino acid region. The amino acid residues shown in each chimeric gene represent the start and end of the substituted region. For constructs with substitutions of individual residues, the substituted amino acids are shown in *bold*. Odd numbered constructs are *S. canaliculatus* Δ4 desaturase sequences with substitutions and even numbered constructs are *S. canaliculatus* Δ5/6 desaturase sequences with substitutions. *S. canaliculatus* Δ4 desaturase sequence is represented as a *white bar* and *S. canaliculatus* Δ5/6 is represented as a *black bar*. The conversion percentages are the mean of three individual assays ±SD

Table 1 Amino acid substitutions introduced into the region of Δ4 and Δ5/6 desaturases critical for substrate specificity

Residue position[a]	1	2	4
Δ4 desaturase	Y to F	N to H	N to Q
Δ5/6 desaturase	F to Y	H to N	Q to N

[a] Residue numbering is based on the Δ4 and Δ5/6 sequences YNYN and FHYQ, respectively. Residues from the Δ4 desaturase were substituted into the corresponding position of the Δ5/6 sequence, and vice versa

the front-end desaturases makes it challenging to identify enzyme regions affecting regiospecificity and substrate preference. Conversely, with more closely related enzymes, construction of chimeric or mutant proteins has been useful to determine the regions responsible for substrate specificity, as demonstrated with studies on Δ6, Δ8, Δ9 and Δ12 desaturases including acyl-acyl carrier desaturases and sphingolipid desaturases [14, 38–41]. For example, in work with soluble fatty acid desaturases having >70 % similarity in amino acid composition, Cahoon et al. [39] were able to pinpoint the region involved in substrate binding, allowing them to modify a Δ6 acyl-acyl carrier protein desaturase active on 16:0 fatty acids to function as a Δ9 acyl-acyl carrier protein desaturase active on 18:0. The critical amino acids appeared to be located either on the surface of the enzyme pointing away from the substrate binding channel or away from the active site, based on the predicted schematic model. Hence, the authors suggested the change in the position of double bond insertion was due to subtle packing effects in the enzyme rather than direct interactions with the substrate. Similarly, comparisons between highly similar acyl-acyl carrier protein thioesterases from *Cinnamomum camphorum* and *Umbellularia californica* active on 12:0 and 14:0, respectively, allowed Yuan et al. [38] to identify three amino acids which were responsible for substrate specificity, and to modify the *Umbellularia californica* 12:0 acyl-acyl carrier protein thioesterase to accommodate 14:0 substrate.

Thus, the unusually high identity in amino acid composition (83 %) between *S. canaliculatus* Δ4 and Δ5/6 desaturases gave us an advantage in locating individual regions or residues involved in substrate specificity and regioselectivity. We identified a discrete protein region containing just four amino acid residues (YNYN at position 280–283 on *S. canaliculatus* Δ4 desaturase and the corresponding amino acids FHYQ at position 278–281 on the *S. canaliculatus* Δ5/6 desaturase; Fig. 1) which influences the both substrate chain-length specificity and regioselectivity of *S. canaliculatus* Δ4 and Δ5/6 desaturases (Fig. 5b). The absence of a crystal structure for any membrane bound desaturase makes it somewhat difficult to structurally interpret the alteration in regioselectivity and substrate specificity observed here. However, the *S. canaliculatus* Δ4 and Δ5/6 desaturases are membrane bound enzymes, and based on previous studies the histidine boxes as well as the N- and C-termini of such enzymes have been predicted to be located on the cytoplasmic side of the membrane. Given these constraints, the membrane protein topology prediction software (TOPCONS [32]) predicts that the *S. canaliculatus* desaturases have four transmembrane domains (amino acid residues 132–152, 159–179, 265–285, 303–323 for Δ4 and corresponding residues for Δ5/6) linked by two exoplasmic regions consisting of six and seventeen amino acids (Fig. 1). It is expected that these highly similar desaturases would share a common membrane topology and presumably a common structural folding.

The four critical amino acids influencing the substrate specificity and regioselectivity of the *S. canaliculatus* desaturases are predicted to be located in the third putative transmembrane domain (Fig. 2). This four amino acid residue block is some distance from the histidine rich active site, which suggests that no direct interaction between the critical amino acid residues and the substrate are required for the observed alterations in regiospecificity and substrate specificity [39]. However, these four amino acid residues may form part of a hydrophobic substrate binding pocket which places constraints on the chain length of the substrate. Influences due to modification of the hydrophobic substrate binding pocket were observed in lipoxygenases and soluble fatty acid desaturases, and it was suggested that the size and geometry of this hydrophobic binding pocket influenced the substrate specificity and the positions at which double bonds were inserted [39, 42]. The amino acid residues YNYN in the critical region of the *S. canaliculatus* Δ4 desaturase are somewhat less bulky than the respective FHYQ residues in *S. canaliculatus* Δ5/6 desaturase, possibly allowing the hydrophobic substrate binding pocket to accommodate a larger substrate such as DPA, whereas the presence of bulkier residues may put a greater constraint on the substrate binding pocket to favor the smaller substrate ALA. Alternatively, the presence of a

histidine residue in proximity to aromatic amino acids may result in hydrogen-π and π–π stacking interactions between aromatic amino acids which could influence the protein interaction and thus affect the substrate preference [43]. The putative localization of the four critical amino acids in the third transmembrane domain concurs with the findings of Libisch et al. [14] who proposed that the transmembrane domains of the *Borago officinalis* Δ6 desaturase are involved in substrate chain length selectivity.

In this study, we have generated novel enzymes with both Δ 4 and Δ6 desaturase activities (Sig-22 to 26C; Fig. 5b) by altering only the first or second amino acids in a critical four-amino acid region (Table 1). The dual enzyme activities of the mutants were unexpected, and substituting both residues from a given desaturase increased activity toward the substrate of that desaturase by 2.4–3.3 fold compared to altering only the first amino acid. A single amino acid substitution at the fourth position in this region (Table 1), results in a lower catalytic ability in both Δ4 and Δ5/6 desaturases but does not affect the substrate or double bond position specificities. However, when this substitution was combined with double amino acid substitutions at positions 1 and 2 (Table 1), the Δ4 desaturase was converted to an enzyme with Δ5/6 desaturase activity and substrate specificity, while the Δ5/Δ6 desaturase lost activity with ALA and showed a low level of Δ4 desaturase activity. This indicates that the substrate specificity and regioselectivity of the *S. canaliculatus* desaturases cannot be confined to a single amino acid residue. In fact, some degree of interaction between all four amino acid residues (Δ4: YNYN and Δ5/6: FHYQ) appears to be responsible for the substrate specificity and regioselectivity of the enzymes. Interestingly, similar results were obtained by Yuan et al. [38]. A single amino acid change (T231K) in a acyl-acyl carrier protein thioesterase by itself did not affect substrate specificity but when this mutation was added to an enzyme with two mutations (M197R/R199H) which possessed equal preference for 12:0 and 14:0, the triple mutant was active only on 14:0. The single mutation at position 4 (Table 1) shows a nonadditive combinatory effect on the double mutant at positions 1 and 2, which suggests that some type of interaction is occurring between these amino acids [38, 44].

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Conflict of interest There is no conflict of interest for this study.

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