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Mutation study of conserved amino acid residues of *Spirulina* Δ^6-acyl-lipid desaturase showing involvement of histidine 313 in the regioselectivity of the enzyme

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Abstract In the cyanobacterium *Spirulina platensis*, the desaturation process is carried out by three desaturases: the Δ^9, Δ^{12} and Δ^6 desaturases, encoded by *desC*, *desA* and *desD*, respectively. The Δ^6 desaturase is responsible for the catalysis of linoleic acid, yielding γ-linolenic acid ($18{:}3^{\Delta 9,12,6}$), the end-product of the process. In this study, the *desD* gene was expressed in *Escherichia coli* using a pTrcHisA expression system. In order to identify the amino acid residues involved in the enzymatic activity, a sequence comparison was performed using various organisms. The alignment revealed three conserved histidine clusters, a number of conserved residues among all listed organisms and a few conserved residues among cyanobacterial species possibly involved in the desaturation activity. A series of site-directed mutations were generated in the *desD* gene to evaluate the role of these residues vis-à-vis the enzyme function. This approach revealed that: (1) H313 is involved in the regioselectivity of the enzyme, (2) the three histidine clusters together with H313, H315, D138 and E140 are required for enzymatic activity, most likely as providers of the catalytic Fe center and (3) W294 is also essential for the activity of Δ^6 desaturase, possibly by forming part of the substrate-binding pocket.

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Introduction

Desaturases are known for their ability to catalyze the formation of unsaturated fatty acids. These enzymes act by introducing a double bond at a specific defined position in the hydrocarbon chain (Holloway 1983) and are grouped into three types. Cyanobacterial fatty acid desaturases are categorized as being part of the family of acyl-lipid desaturases or plant-type desaturases (Murata and Wada 1995), which are membrane-bound enzymes (Mustady et al. 1996). Acyl-lipid desaturases introduce double bonds into fatty acids that have been esterified to glycerolipids (Schmidt et al. 1993).

The Δ^6 desaturase of *Spirulina platensis* converts linoleic acid (18:2) to γ-linolenic acid (GLA). The isolation of the *desD* gene encoding the Δ^6 desaturase of *S. platensis* C1 was reported by Murata et al. (1996). A study conducted by our group on the regulation of desaturase gene expression in *S. platensis* revealed a 30% increase in the level of γ-linolenic acid after reducing the growth temperature from 35°C to 22°C (Deshnium et al. 2000). We also found that this enzyme is present in both the plasma and the thylakoid membranes and that the response to immediate temperature reduction differs in accordance with the membrane in question (Hongsthong et al. 2003).

The presence of conserved histidine clusters has been reported in other membrane-bound desaturases in mammals, fungi, insects, higher plants and cyanobacteria (Bloomfield and Bloch 1960; Avelange-Macherel et al. 1991; Diaz et al. 2002). Site-directed mutagenesis studies carried out with these conserved histidine residues (Shanklin et al. 1994) demonstrate that they play a crucial role, possibly by providing iron-active sites (Sundberg and Martin 1974). As a consequence, membrane-bound desaturases are classified into a superfamily of membrane iron proteins. It is also proposed that the hydrophobic stretch located between the two first histidine clusters and the other, positioned closer to the third histidine cluster, might be involved in substrate recognition (Diaz et al. 2002).

Integral membrane or membrane-bound desaturases, including *Spirulina* desaturases, are less well understood than soluble desaturases, due to the difficulties in obtaining large quantities of purified membrane-bound proteins (Shanklin and Cahoon 1998). A biochemical study of the *Spirulina* desaturases is one of the keys to understand how the cells survive under low-temperature conditions, when the cells synthesize more of the end-product of desaturation: GLA.

In many studies, such as those involving the analysis of an enzyme-active site with regioselectivity and substrate specificity, a site-directed mutagenesis approach has been applied to accomplish these goals (Cahoon et al. 1997a, b; Mcguire et al. 2001). In the present study, to explore the role of the residues involved in the catalytic activity of Δ^6 desaturase, we conducted site-directed mutagenesis, substituting the histidine residues in the tripartite conserved histidine motif with arginine. The side-chain of histidine is an imidazole group (pK_a=6), which can donate a proton to serve as a ligand to chelate metal ions. The arginine side-chain, in contrast, is a guanidinium group (pK_a=12.5), which is a strong base. The evolutionarily conserved residues of Δ^6 desaturase in all organisms listed in Fig. 1 (D138 and W294, which have the most acidic and the most complex aromatic side-chain, respectively) were changed to N and G (which have the most basic and the most simple side-chain, respectively). In addition, other evolutionarily conserved residues among the cyanobacteria (R123, G136, E140, H313, H315) were substituted with N, H, Q, R and N, found at the corresponding position of *Mucor rouxii*, *Borago officinalis* or *Homo sapiens*.

The expression of mutant enzymes was detected by Western blot analysis; and the effects of mutation were analyzed by determination of the end-product. The analyses of the mutant forms of the enzyme permitted classification into three groups: (1) those with an undetectable level of Δ^6 desaturase activity and the presence of an unusual fatty acid, (2) those with an undetectable level of Δ^6 desaturase activity and (3) those with reduced Δ^6 desaturase activity. The resulting data confirmed the hypothesis that the three conserved histidine motifs play a crucial role in enzymatic activity, most probably by building the iron active site. Moreover, the results demonstrated the requirement of a number of conserved amino acid residues, in addition to the three histidine motifs, for the enzymatic function. Interestingly, the single substitution mutation of H313 with arginine caused an alteration in the fatty acid desaturation position of the enzyme.

Materials and methods

Bacterial strains and plasmids

The bacterial strains used in this study were *Escherichia coli* DH5α chemically competent cells and *E. coli* XL-mut-S and XL-1-Blue competent cells. The plasmid pTrcHisA (Invitrogen, USA) was used for cloning the *desD* gene for the construction of an expression vector in *E. coli*. *E. coli* strains were grown routinely at 37°C in Luria–Bertani broth (LB) or LB agar (Sambrook et al. 1989), supplemented with appropriate antibiotics when necessary.

Chemicals and enzymes

All the chemicals used for the in vitro assay of enzyme activity were purchased from Sigma (USA), while the enzymes for DNA manipulations were purchased from New England Biolabs (USA), Promega (USA) and Invitrogen (USA) and used according to the manufacturers' instructions. A Chameleon double-stranded site-directed mutagenesis kit from Stratagene (USA) was used for generating site-directed mutations in the *desD* gene.

DNA manipulation

Plasmid DNA from *E. coli* was isolated using the alkaline lysis method (Sambrook et al. 1989). DNA sequences were determined using standard methodologies for double-stranded plasmid DNA on an automated DNA sequencer (Applied Biosystem, USA) at the Bio Service Unit, National Science and Technology Development Agency, Bangkok, Thailand. The analysis of DNA sequences was carried out using the Genetyx package program.

Construction of plasmid for *desD* gene expression in *E. coli*

The open reading frame of the Δ^6 desaturase of *S. platensis* C1 was amplified from the *desD* clone (Murata et al. 1996) by PCR using two oligonucleotide primers, the synthesis of which was based on the *desD* coding sequence. The two oligonucleotide primers used for PCR amplification were the *desD* forward primer (5′-TGG*GGATCC*TTAATGACATCAACAAC-3′) containing the *Bam*HI restriction site (italics) upstream of the start codon, together with the *desD* reverse primer (5′-GG*GAAGCT*TAAGGTTAGTGATGATT-3′) containing the *Hin*dIII restriction site (italics) downstream of the stop codon. The amplification of DNA fragments was performed using taq DNA polymerase and a corresponding buffer (Invitrogen, USA) in a final volume of 100 μl in a DNA thermal cycler (Bio-Rad). The amplification process began with 3 min at 94°C, followed by 30 cycles of 94°C for 40 s, 50°C for 1 min and 72°C for 2 min, concluding with a final extension at 72°C for 10 min. The PCR-amplified products were digested with *Bam*HI and *Hin*dIII, followed by gel purification. After elution, the respective DNA fragments were subcloned into the pTrcHisA expression vector, thus generating plasmid pTrcHisA-*desD*. This plasmid was transformed into *E. coli* DH5α host cells using the $CaCl_2$ transformation

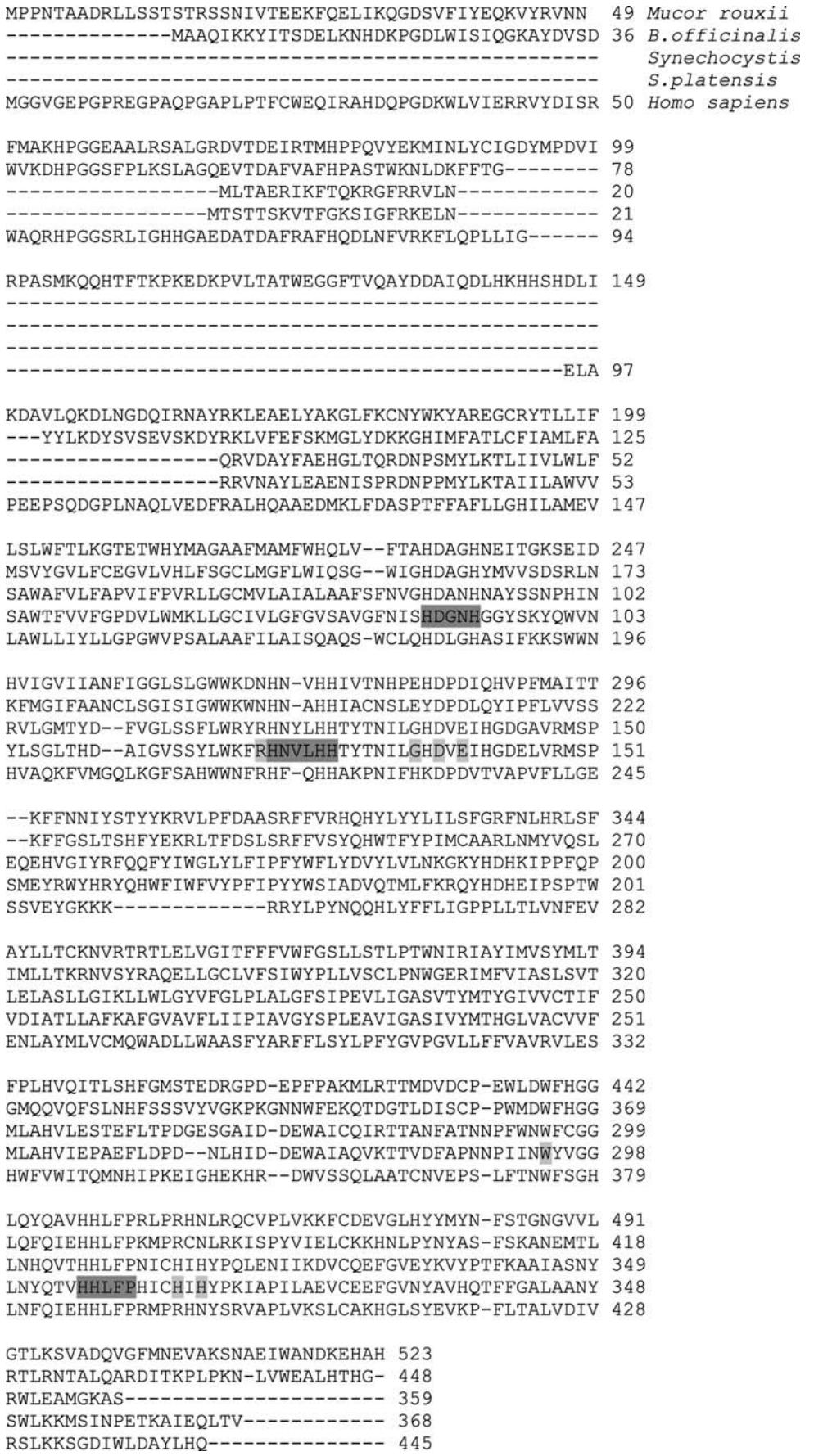

Fig. 1 Comparison of the deduced amino acid sequence of Δ^6 desaturase from *M. rouxii*, *B. officinalis*, *Synechocystis*, *Spirulina platensis* and *H. sapiens*. The signature motifs common to fatty acid desaturase are highlighted in *dark gray*, while the other residues subjected to mutagenesis are highlighted in *light gray*

method (Sambrook et al. 1989) for Δ^6 desaturase expression studies.

Construction of site-directed mutants

Single- and double-point mutations encoding single amino acid substitutions were introduced directly into pTrcHisA-*desD*. Site-directed mutagenesis was carried out using the method employed by Deng and Nickoloff (1992), with the Chameleon double-stranded site-directed mutagenesis kit (Stratagene, USA) used according to the manufacturer's instructions. Two synthetic oligonucleotide primers were used for the desired mutation. One primer (henceforth referred to as the mutagenic primer) was used to introduce the desired mutation, while the other (the selection primer) was used to mutagenize a unique restriction site in the plasmid for subsequent selection. The nucleotide sequences of the mutagenic primers used for mutagenesis are shown in Table 1. The mutations were H89R, H93R, H124R, H128R, H129R, H305R, H306R (conserved histidine residues of three histidine motifs), R123N, G136H, E140Q, W294G, H313R, H315N, D138N (conserved amino acids outside the histidine motifs). The letters in italics and underlined indicate altered nucleotides and codons, respectively. All the mutations were verified by sequencing. The resulting plasmids were used for *E. coli* transformation.

Culture conditions

E. coli DH5α was used as the host for expression of the *desD* gene. Cells containing pTrcHisA-*desD* were grown in LB in the presence of 100 μg ml^{-1} of ampicillin at 37°C, and shaken at 200 rpm. In order to assay Δ^6 desaturase activity in vitro, *E. coli* DH5α was grown in M9 medium supplemented with 4 mg ml^{-1} glucose, 0.1 mM $MgSO_4$, 1 mg ml^{-1} casamino acids, 10 μM $FeCl_3$, 0.5 μg ml^{-1} vitamin B1, 100 μg ml^{-1} ampicillin (Wada et al. 1993) and sodium linoleate (a substrate of Δ^6 desaturase) at concentrations of 0, 100, 200, 300, 400, 500, 600, and 800 μM. The culture was incubated at 37°C and shaken at 200 rpm. When the optical density of the culture at 600 nm reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to yield a final concentration of 1 mM. The culture was further incubated at 30°C and shaken at 200 rpm for 3 h, until the optical density of the culture at 600 nm reached 0.7–0.75. The cells were harvested by centrifugation at 9,000 rpm for 10 min. The pellet was then washed twice with 30 ml of re-suspended buffer containing 50 mM MOPS-NaOH, pH 7.5, 10 mM $MgCl_2$ and stored at −20°C until used.

In vitro assay

In a previous study, we showed that Δ^6 desaturase cannot function in vivo (data not shown), possibly due to the absence of essential cofactors in the host cells. We thus modified the method employed by Wada et al. (1993) to conduct an in vitro assay in order to detect the enzymatic activity of *Spirulina* Δ^6 desaturase expressed in *E. coli*.

The cell pellet was re-suspended in buffer containing 50 mM MOPS-NaOH, pH 7.5, 10 mM $MgCl_2$, 300 mM sorbitol, 10 ng of DnaseI and 10,081 units of catalase (Wada et al. 1993). The cells were disrupted by passage through a chilled French pressure cell operated at 3.45 MPa, with this process repeated three times in order for each sample to achieve complete disruption. The cell debris was then removed by centrifugation at 10,000 rpm for 10 min at 4°C and the homogenate immediately subjected to the assay for Δ^6 desaturase activity. An aliquot of the homogenate corresponding to 2–4 mg of total protein (quantified using Lowry's 1951 method) was mixed with the following components in a total volume of 1.2 ml: 40 mM tricine-KOH, pH 8.0, 10 mM $MgCl_2$, 100 μg of ferredoxin (F3013, Sigma), 5 mM β-NADPH (N1630, Sigma), 50,405 units of catalase (C3155, Sigma) and 200 milliunits of Fd–$NADP^+$ oxidoreductase (F0628, Sigma). The reaction was incubated at room temperature (approximately 25°C)

Table 1 Synthetic oligonucleotide primers used for site-directed mutagenesis. Codons for the changed amino acids are underlined, while the letters in italics indicate the altered nucleotides

Mutation	Mutagenic oligonucleotide sequence (5′– 3′, Sense)
H89R	GGCCGTCGGTTTTAACATTAGC*CGT*GACGGAAATCATGGAGG
H93R	GCCATGACGGAAAT*CGT*GGAGGTTATTCTAAATATCAATGGGTG
H124R	TATGGAAGTTTCGC*CGC*AATGTACTTCATC
H128R	GCCACAATGTACTT*CGT*CATACCTACACCA
H129R	CACAATGTACTTCAT*CGT*ACCTACACCAACA
H305R	CTATCAAACCGTC*CGC*CATCTATTTCCTC
H306R	ATCAAACCGTCCAC*CGT*CTATTTCCTCATATC
R123N	ATGGAAGTTT*AAC*CACAATGTACTTCATCATACC
G136H	CCTACACCAACATTTTA*CAT*CATGATGTGGA
D138N	CATTTTAGGTCAT*AAT*GTGGAAATTCATG
E140Q	TAGGTCATGATGTG*CAA*ATTCATGGGGAT
W294G	CCAATTATTAAC*GGG*TATGTAGGGGGA
H313R	TCCTCATATCTGC*CGC*ATCCATTATCCTA
H315N	CCTCATATCTGCCACATC*AAT*TATCCTAAAATTGCTCC

for 15 min—the appropriate time for incubation according to time-course studies (data not shown). The lipid was then extracted from the reaction mixture and analyzed using gas chromatography.

The substrate used in this assay was $18{:}2^{\Delta 9,12}$ in the form of a sodium salt. Since the desaturases of *S. platensis* are acyl-lipid desaturases, the free fatty acids taken up must be incorporated into glycerolipids, the form of substrate that these desaturases can utilize. In general, *E. coli* is able to incorporate exogenously added fatty acids into glycerolipids (Esfahani et al. 1969), so the substrate must be added to the culture medium.

Western blot analysis

Detection of the Δ^6 desaturase was achieved by Western blot analysis (Gravel and Golaz 1996), using a monoclonal antibody against the 6× histidine tag, as described by the manufacturer (Amersham Biosciences, Sweden). The protein samples, which were separated by 12% SDS-PAGE, were transferred onto a nitrocellulose membrane using a semi-dry electroblotter (Bio-Rad) at a constant voltage of 20 V for 30 min at room temperature. A Western blot detection kit with an alkaline-phosphatase detection system was used (Zymed, USA) as recommended by the manufacturer.

Fatty acid extraction and analysis

After the lipid was extracted from the assay mixture, the extract was transmethylated using 5% HCl in methanol and stirred in the dark at 85°C for 150 min (Wada et al. 1993). The methylated fatty acids were then separated and analyzed using gas chromatography (Shimadzu GC 17-A). The capillary column used in the analysis was a fused silica glass column (30 m, OMEGAWAX 250; Supelco, USA) with a film thickness of 0.25 μm. The injector temperature was 205°C and the split ratio was 1:10.

The positions of the double bonds of the fatty acids were identified by 4,4-dimethyloxazoline (DMOX) derivatization prior to analysis by GC-MS (Fay and Richli 1991).

GC-MS analysis

The qualitative analysis of the fatty acid products, synthesized by heterologously expressed desaturases, was conducted using a TRACE GC/PolarisQ GC-MS (Thermo Finnigan, USA) in order to assure the presence of the products. GC was performed on a fused silica glass column (30 m, OMEGAWAX 250; Supelco, USA) with a film thickness of 0.25 μm. The column temperature, flow rate and split ratio were 205°C, 1.0 ml min^{-1} and 1:10, respectively. The GC was directly interfaced (with a transfer-line temperature of 300°C) to a PolarisQ mass selective detector (Thermo Finnigan) run in full-scan mode. The electron beam energy was 70 eV. Qualitative analysis was performed using a library search. The libraries used in this experiment were the National Institute of Standards and Technology library and a library constructed using standard fatty acids (Sigma, USA). The analysis of these standards was performed on this instrument under the conditions described.

The double-bond position of the fatty acids synthesized by the heterologously expressed desaturases was determined by GC-MS analysis of the DMOX derivative of the fatty acids in question. The GC-MS conditions were as follows: a fused silica glass column (30 m, OMEGAWAX 250; Supelco) with a film thickness of 0.25 μm, an oven temperature program at 80°C (3 min), increasing at 20°C min^{-1} to 180°C (15 min) and then at 2°C min^{-1} to 280°C (15 min), an ion-source temperature of 200°C and ionization at 70 eV.

Results

Detection of Δ^6 desaturase expressed in *E. coli*

The Δ^6 desaturase expressed in *E. coli* using the pTrcHisA system contained a 6× histidine tag at the N-terminus of the polypeptide. Detection was achieved by Western blot analysis, using a monoclonal antibody against the 6× histidine tag. Results showed that the Δ^6 desaturase was present in all mutants (Fig. 2) with an approximate molecular mass of 47 kDa.

In vitro assay of Δ^6 desaturase

In general, *E. coli* is able to incorporate exogenously added fatty acids into glycerolipids (Esfahani et al. 1969).

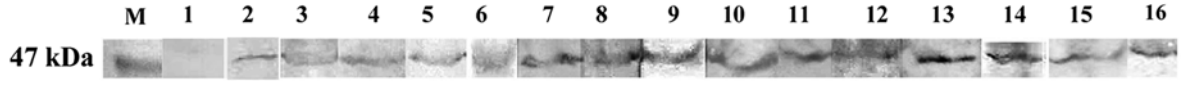

Fig. 2 Detection of the heterologously expressed Δ^6 desaturase from pTrcHis-*desD* and its mutants. Total cell extracts were prepared and total protein (15–20 μg protein per lane) was analyzed as described in the Materials and methods. *M* Pre-stained molecular weight markers. *Lane 1* pTrcHisA, *lane 2* pTrcHis-*desD*, *lane 3* pTrcHis-$desD^{H89R}$, *lane 4* pTrcHis-$desD^{H93R}$, *lane 5* pTrcHis-$desD^{H124R}$, *lane 6* pTrcHis-$desD^{H128R}$, *lane 7* pTrcHis-$desD^{H129R}$, *lane 8* pTrcHis-$desD^{H305R}$, *lane 9* pTrcHis-$desD^{H306R}$, *lane 10* pTrcHis-$desD^{H313R}$, *lane 11* pTrcHis-$desD^{H315N}$, *lane 12* pTrcHis-$desD^{R123N}$, *lane 13* pTrcHis-$desD^{G136H}$, *lane 14* pTrcHis-$desD^{E140Q}$, *lane 15* pTrcHis-$desD^{W294G}$, *lane 16* pTrcHis-$desD^{D138N}$

When the host cells containing the designated plasmid with the insert are grown in the presence of sodium-linoleic acid, they can take up the exogenous substrate and transform it into a glycolipid, the only form that the *Spirulina* acyl-lipid Δ^6 desaturase can utilize (Mustady et al. 1996). Also, it has been reported that cyanobacterial desaturation reactions require the reduced form of ferredoxin (Tocher et al. 1998). We therefore conducted our in vitro assay of the cell extract containing a substrate in the form of glycolipid and either Δ^6 desaturase or modified Δ^6 desaturase in the presence of exogenously provided cofactors. The resulting product was subsequently identified by GC (Fig. 3). In addition, it should be noted that GC-MS analysis of the two unknown peaks appearing after the peak of GLA in Fig. 3b was performed and the results showed that these peaks are not α-linolenic acid (ALA).

Site-directed mutagenesis of Δ^6 desaturase

Mutations of histidine residues in the three conserved histidine clusters that are purportedly involved in providing the catalytic iron center should theoretically diminish the ability of the enzyme to bind iron and thus eliminate desaturase activity (Murata and Wada 1995). To determine the role of these three histidine motifs in Δ^6 desaturase, the mutated *desD* gene was expressed in *E. coli*, with the assay carried out in vitro. The resulting product was then analyzed.

The amino acid sequence alignment shown in Fig. 1 demonstrates that 14 amino acid residues were changed one-by-one using a Chameleon site-directed mutagenesis kit. Nine histidine residues were selected. Seven residues (H89, H93, H124, H128, H129, H305, H306) within the three conserved histidine motifs were mutated to arginine. In addition, two residues (H313, H315, which are conserved among cyanobacterial species and located adjacent to the third cluster) were mutated to arginine and asparagine, respectively. All the site-directed mutations of histidine residues within the three clusters caused an 80–100% reduction in the in vitro activity (Table 2); and mutation of the two conserved histidine residues (H313, H315) showed a complete elimination of desaturase activity (Table 2).

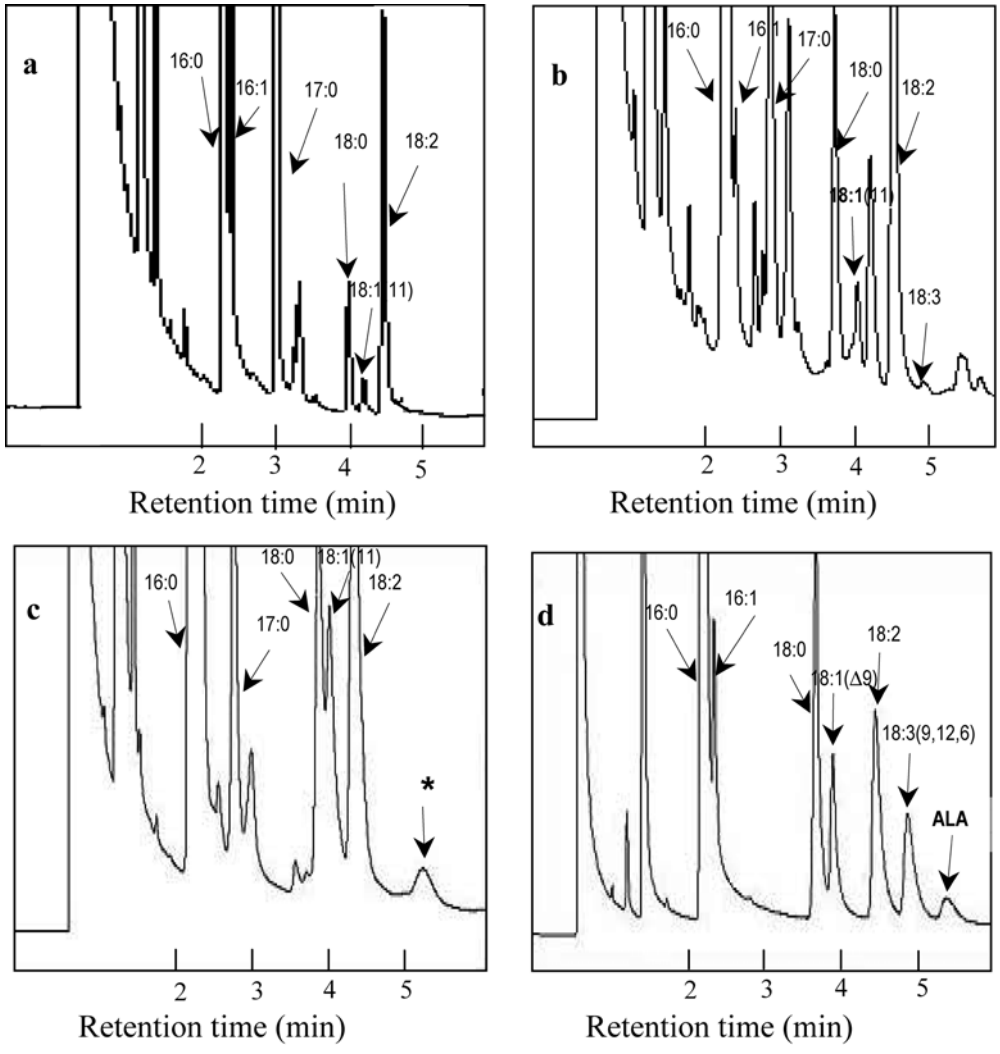

Fig. 3 GC profiles of total fatty acids extracted from **a** *E. coli* containing pTrcHisA (control), **b** pTrcHis-*desD* and **c** pTrcHis-*desD*^{H313R} in the presence of the substrate 18:2$^{\Delta 9,12}$; and GC profile of **d** standard fatty acids, including ALA. The *asterisk* indicates the unusual fatty acid peak found in pTrcHis-*desD*^{H313R}

Table 2 Specific activity of the modified Δ^6 desaturase obtained from site-directed mutagenesis in the presence of 300 μM sodium linoleate. Values represent the average of three independent experiments. The deviation of the specific activity is within ±0.02. The specific activity of wild-type *Spirulina* Δ^6 desaturase expressed in *E. coli* in the presence of 300 μM sodium linoleate was 3.6 mg GLA mg protein^{-1} h^{-1}

Residue location	Residues changed	Specific activity (mg GLA mg protein^{-1} h^{-1})	% activity remaining (compared with wild-type enzyme)
First His cluster	H89R	0	0
First His cluster	H93R	0.4	11
Second His cluster	H124R	0	0
Second His cluster	H128R	0	0
Second His cluster	H129R	0	0
Third His cluster	H305R	0.6	17
Third His cluster	H306R	0	0
Outside His cluster	H313R	0	0
Outside His cluster	H315N	0	0
Outside His cluster	R123N	3.3	91
Outside His cluster	G136H	0	0
Outside His cluster	E140Q	0	0
Outside His cluster	W294G	0	0
Outside His cluster	D138N	0	0

Interestingly, one of these mutants (H313R) revealed the synthesis of an unusual fatty acid with the same retention time as that of ALA (18:3$^{\Delta 9,12,15}$; Fig. 3c,d). GC-MS analysis of the methyl-ester derivative of the fatty acid revealed that it was likely to be ALA, a conclusion drawn from the fact that the product obtained from the mutant enzyme possessed *m*/*z* 292, the parent ion of 18:3 methyl-ester. Moreover, analysis of the DMOX derivative of the unusual fatty acid synthesized by the H313R mutant enzyme showed *m*/*z* 196, 208, 236, 248, 276 and 288 (Fig. 4a), indicating double-bond positions at Δ9, Δ12 and Δ15, using the rule of 12 atomic mass unit intervals (Fay and Richli 1991). Analysis of the DMOX derivative fatty acid synthesized by the heterologously expressed Δ^6 desaturase, however, showed *m*/*z* 194, 206, 234, 246, 220, 274, 166 and 167, indicating double-bond positions at Δ6, Δ9 and Δ12 (Fig. 4b).

In addition, since the H313R mutant enzyme has the ability to introduce a double bond at the Δ15 position, 10 μmol of GLA (18:3$^{\Delta 9,12,6}$) was added to the culture medium of *E. coli* containing pTrcHis-*desD*^{H313R}. The substrate was in the form of free fatty acid, due to the fact that GLA in the form of a sodium salt is not commercially available. The data showed that: (1) the cells were able to take up the substrate in the form of free fatty acid and (2) the mutant enzyme produced an extremely small peak of fatty acid product, which had a higher retention time than that of GLA. Then, a GC-MS analysis of the fatty acid was performed and the spectrum showed that this fatty acid was possibly stearidonic acid (SDA, 18:4$^{\Delta 9,12,6,15}$) due to the presence of *m*/*z* 166, 167, 194, 206, 220, 234, 246, 260, 274 and 286 (Fig. 4c).

The single mutation of residue G136, E140, W294 or D138 totally eliminated the Δ^6 desaturase activity, whereas mutation of R123 resulted in activity close to that of the wild type in the presence of 300 μM substrate (Table 2). However, the K_m and V_{max} of R123N (defined using a Lineweaver–Burk plot) were found to be 500 μM and 5 mg GLA mg protein^{-1} h^{-1}, respectively. This mutation of a positively charged and non-polar residue (arginine) to an uncharged and polar residue (asparagine) led to a reduction in V_{max} by approximately 50%, while the amount of K_m remained unaltered (Table 3).

Discussion

This study was performed with the aim of identifying those amino acid residues within the *Spirulina* Δ^6 desaturase required for its catalytic activity. The amino acid sequence alignment of various groups of Δ^6 desaturase from various organisms demonstrates three histidine

Table 3 Kinetic properties of *Spirulina* acyl-lipid Δ^6 desaturase and mutant R123N using sodium linoleate (18:2$^{\Delta 9,12}$) as the substrate. The kinetic constants (K_m and V_{max}) were defined using a Lineweaver–Burk plot

Δ^6 desaturase	Kinetic parameter		
	K_m (μM)	V_{max} (mg GLA mg protein^{-1} h^{-1})	V_{max}/K_m (mg GLA mg protein^{-1} h^{-1} μM^{-1})
Wild type (DesD)	500	10	0.02
DesD-R123N	500	5	0.01

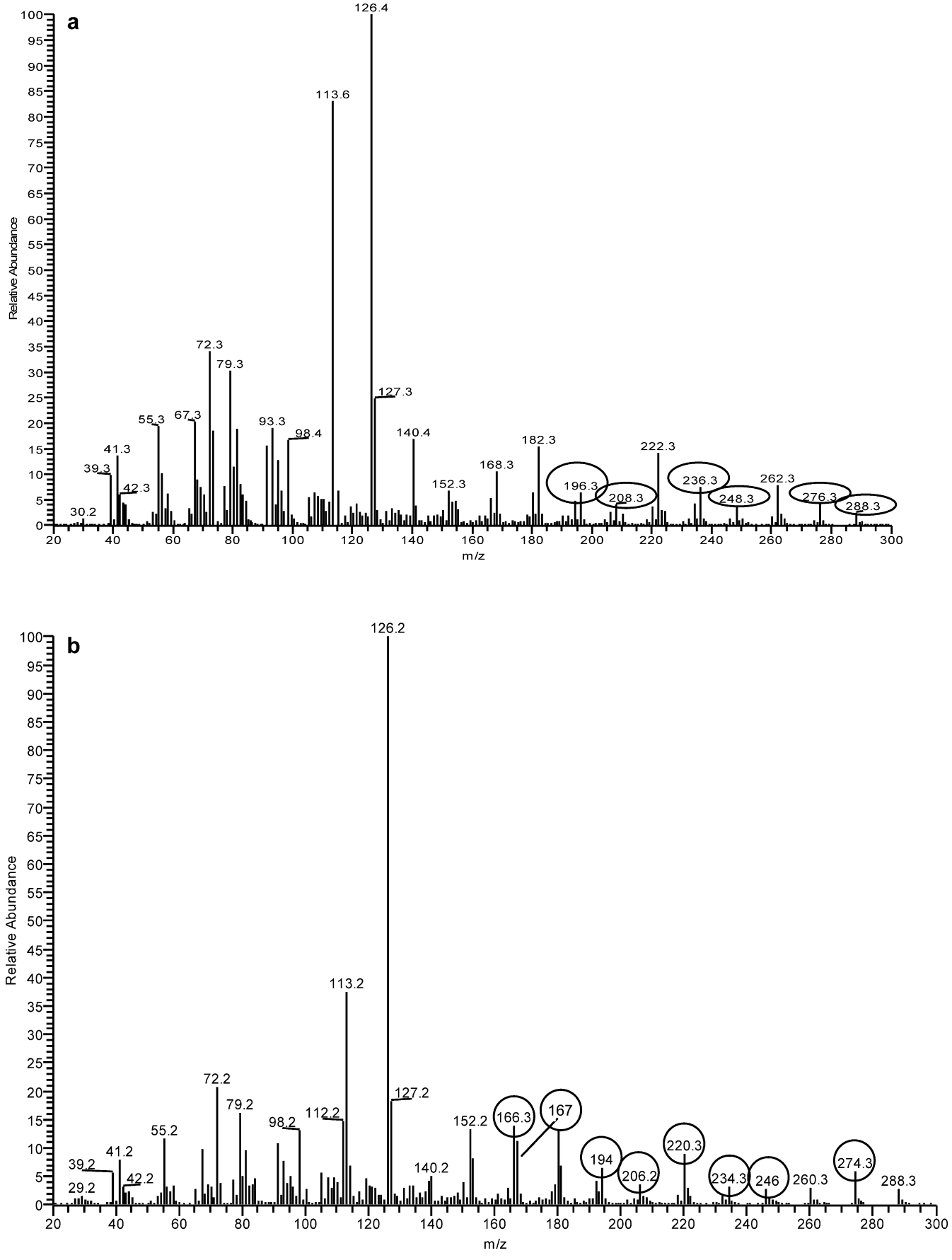

Fig. 4 Mass spectrum obtained from the GC-MS analysis of the DMOX derivative of **a** the unusual fatty acid extracted from pTrcHis-*desD*^{H313R}, **b** the fatty acid GLA extracted from pTrcHis-*desD* in the presence of linoleic acid (18:2$^{\Delta 9,12}$) and **c** the unusual fatty acid extracted from pTrcHis-*desD*^{H313R} in the presence of linolenic acid (18:3$^{\Delta 9,12,6}$)

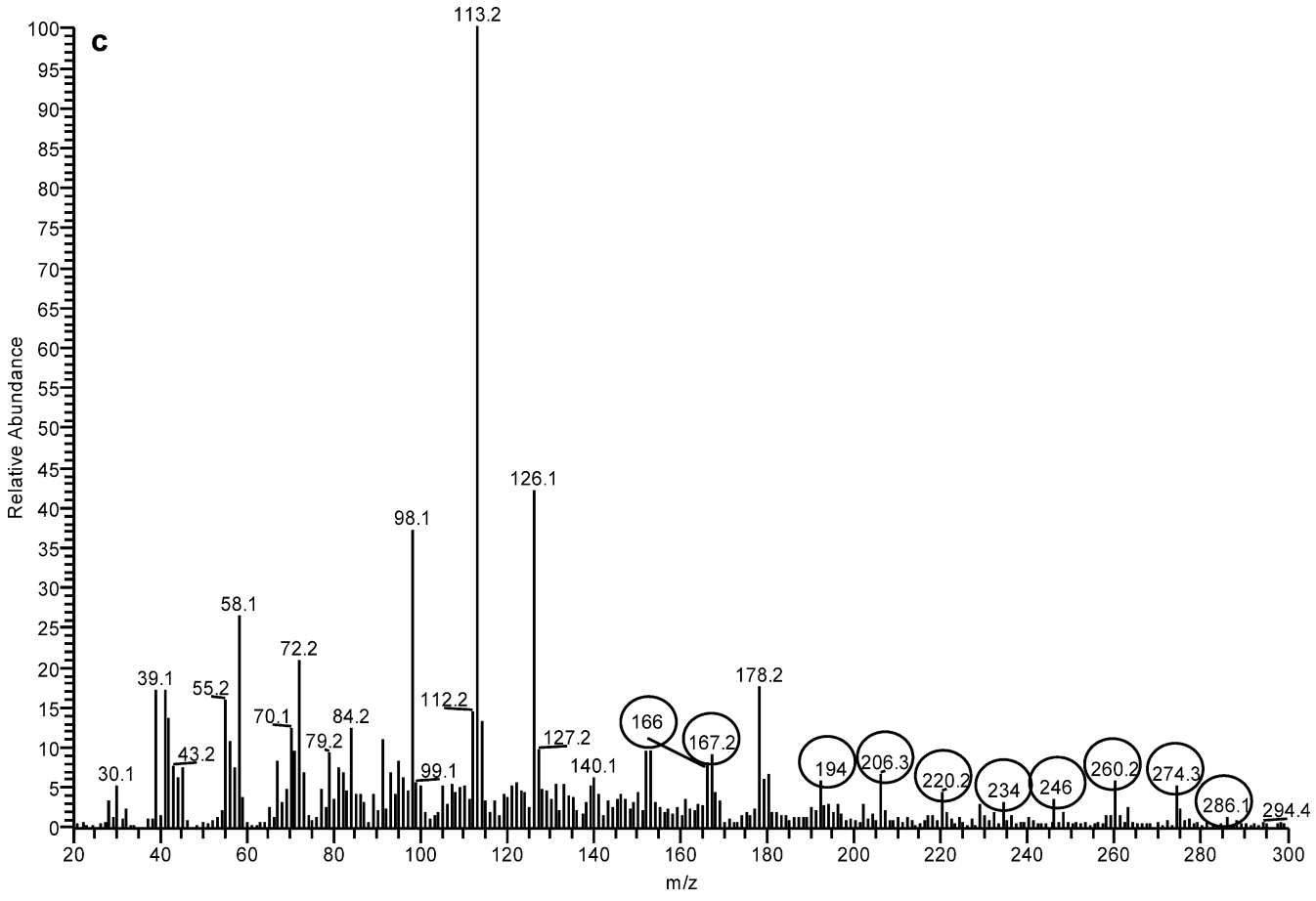

Fig. 4 (continued)

motifs conserved among all Δ^6 desaturases, together with two histidine residues adjacent to the third cluster which are conserved among the acyl-lipid desaturases of cyanobacteria. In addition, there is a set of residues which we propose are located in the cytoplasmic phase and can be divided into three groups: (1) G135 and E140, conserved among cyanobacterial species, (2) D138 and W294, conserved among all listed organisms and (3) R123, conserved among cyanobacterial species and *H. Sapiens* (Fig. 1).

The *Spirulina* Δ^6 desaturase was expressed as approximately 2% of the total protein in *E. coli*. Prior to the assay for enzyme activity being carried out in vitro, it was performed in vivo followed by GC analysis for the enzyme product, GLA. The enzyme product was not detected. However, the fatty acid GLA was detected after the in vitro assay in the presence of exogenously provided cofactors, including a photosynthetic form of ferredoxin from spinach. This indicated that the heterologously expressed enzyme was in a functional form. This finding also corresponded to studies involving desaturase activity influenced by ferredoxin.

In *E. coli*, an extremely low level of ferredoxin protein was expressed, as approximately 0.05% of the cell protein (Ta and Vickery 1992). Thus, an in vivo system for the characterization of desaturase activity in *E. coli* was developed by the co-expression of the ferredoxin gene in this organism (Cahoon et al. 1996). Otherwise, the activity would have to be assayed by the in vitro system. Moreover, Schultz et al. (2000) studied the influence of two isoforms of ferredoxin on the plant acyl–acyl carrier protein (ACP) desaturase: a photosynthetic form from *Arabidopsis* and spinach and a heterotrophic form from *Impatiens balsamina*. The results revealed that heterotrophic ferredoxins are 10- to 20-fold more effective than photosynthetic ferredoxins. Taking all of these data together, it is clear that ferredoxin plays a critical role in the desaturation activity of various desaturases.

The results obtained in the present study using the site-directed mutagenesis approach reveal that the three histidine-rich motifs are required for Δ^6 desaturase activity, most likely by providing the catalytic iron center. Besides these motifs, there is a small set of histidine residues, H313 and H315, located adjacent to the proposed iron ligand. These residues were also found to be critical for catalytic activity.

Interestingly, an unusual fatty acid was synthesized in substantial quantities during the in vitro assay of the mutant H313R expressed in *E. coli*. This fatty acid has the same retention time as that of standard ALA ($18{:}3^{\Delta 9,12,15}$). It has been reported that *S. platensis* contains a trace amount of ALA (Murata and Nishida 1987). Analysis by

GC-MS showed that this fatty acid has a parent ion of 18:3 methyl-ester, *m*/*z* 292, as with GLA. Conducting a GC-MS analysis of the DMOX-derived fatty acid, we performed a test to locate the double bonds (Fig. 4). The spectrum showed that this unusual fatty acid is in fact ALA, a conclusion drawn from the presence of *m*/*z* 196, 208, 236, 248, 276 and 288 (Fay and Richli 1991), indicating double bonds at Δ9, Δ12 and Δ15 (Fig. 4a). Moreover, this mutant enzyme is also able to introduce a double bond at the Δ15 position in the other fatty acid substrate, GLA, and synthesizes SDA ($18{:}4^{\Delta 9,12,6,15}$), as shown in Fig. 4c. These results strongly suggest that this residue might be involved either in the regioselectivity of the enzyme or in the alteration of enzyme function.

The evolutionary study of desaturases by Sperling et al. (2003) similarly reported that the creation of a new regioselectivity of a desaturase affects the amino acid sequence adjacent to the active site, which forms the substrate channel. Moreover, a study recently performed by Cahoon et al. (1998) showed that the single mutation of L118W causes a shift in the substrate specificity of acyl-ACP Δ^9 desaturase. Whittle and Shanklin (2001) used a combinatorial saturation mutagenesis approach to identify two key residues that play a substantial role in the substrate specificity of Δ^9 ACP desaturase. Broadwater et al. (2002) have meanwhile reported that the substitution of 4–7 residues in *A. thaliana* FAD2 (with desaturation activity) with residues from *Ricinus communis* LFAH (with both hydroxylase and desaturase activity) results in a substantial hydroxylase activity of the mutated FAD2. Interestingly, they also demonstrate that a single mutation of methionine at position 324 to isoleucine can cause a substantial shift in catalytic specificity.

In spite of mutations of the conserved histidine motifs, the Δ^6 desaturase activity was also diminished by the single mutation of residue G136, E140, W294 or D138, indicating the critical role of these residues in the enzyme function. The amino acids E, W and D are known to be capable of binding to metal ligands and forming a metal catalytic center (Creighton 1993); and these residues are also located close to the second histidine cluster. They might thus be involved either in providing the catalytic iron center or in the maintenance of the structural integrity of the active site pocket. A similar finding was reported by Zámocky et al. (2001), who found that the site-directed mutagenesis of the distal active site, a conserved triad of arginine–tryptophan–histidine, revealed that these residues were essential for the catalysis of KatGs (plant peroxidase superfamily).

Moreover, W294, which is conserved in the Δ^6 desaturase of all listed organisms, is positioned within the hydrophobic portion (residues 292–294), located close in sequence to the third histidine-rich region. This residue is located in the soluble portion of the enzyme. The discovery of a hydrophobic stretch within the soluble portion close to the di-iron active site can be expected, as it possibly plays a role in interacting with the acyl chains of membrane lipids (Diaz et al. 2002).

In addition, the substitution of R123, which is conserved among cyanobacterial species, to N, which is found conserved at the corresponding position in *M. rouxii* and *B. officinalis*, led to a reduction of approximately 50% in the level of V_{max}, whereas the level of K_m remained constant in comparison with the wild type. This V_{max}/K_m value points to the fact that the substitution of arginine at position 123 with asparagine causes a 50% reduction in enzyme efficiency, again in comparison with the wild type. Interestingly, Diaz et al. (2002) report that the hydropathy plots of several acyl-lipid desaturases show the presence of a hydrophobic segment located between the first two histidine clusters. They thus proposed that this hydrophobic stretch might be involved in substrate recognition. The R123 of *Spirulina* Δ^6 desaturase is also located between the two first histidine clusters and the substitution of this residue causes a partial deficiency of the enzyme. This demonstrates that R123 is also important for the enzyme function, possibly by playing a part in the active site pocket.

This work provides crucial information on the amino acid residues required for the desaturation reaction of *Spirulina* Δ^6 desaturase and identifies the role of three conserved histidine motifs, based on the results of our experimentation. Our data also allow us to delineate the topology of this membrane-bound desaturase more accurately. The three histidine motifs, as proposed by Murata and Wada (1995) and the amino acids H313, H315, R123, G136, E140, W294 and D138 are critical for desaturase activity and are thus likely to be located on the cytoplasmic phase of the membrane. We propose that these residues play a part in forming the active site, while the tripartite motif also possibly plays a role in providing the iron catalytic center.

The most important result to emerge from this study is the revelation for the first time of the role of H313 in the regioselectivity of the enzyme. At the same time, our work raises questions about the residues involved in regioselectivity and substrate specificity; and we propose that further experiments are conducted to elucidate the residues involved.

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