Site-directed mutagenesis of histidine residues in the Δ12 acyl-lipid desaturase of *Synechocystis*

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Abstract In the cyanobacterium *Synechocystis* sp. PCC 6803, there are four acyl-lipid desaturases that are, respectively, specific to the Δ6, Δ9, Δ12 and ω3 positions of fatty acids. The *desA* gene for the Δ12 acyl-lipid desaturase was modified by site-directed mutagenesis, such that four of the histidine residues that are conserved in the four desaturases and one histidine residue that is not conserved were replaced by arginine, and the mutated *desA* genes were overexpressed in *Escherichia coli*. All of these mutations eliminated the Δ12 desaturase activity. These results demonstrate that the five histidine residues are essential for the activity of the Δ12 desaturase, perhaps by providing the ligands for the catalytic Fe center.

Key words: Acyl-lipid desaturase; Cyanobacterium; Histidine; Overexpression; Site-directed mutagenesis; *Synechocystis*

1. Introduction

The desaturation of fatty acids in plants and animals is catalyzed by several distinct desaturases. Each respective enzyme introduces a double bond at a specific position in the hydrocarbon chain. There are three types of fatty-acid desaturase, namely, acyl-CoA desaturases, acyl-ACP desaturases, and acyl-lipid desaturases [1]. Acyl-ACP desaturases introduce double bonds into fatty acids that are bound to ACP [2]; these enzymes are present in the stroma of plant plastids [2]. Acyl-CoA desaturases introduce double bonds into fatty acids bound to coenzyme A [3]; they are bound to the endoplasmic reticulum in animal cells, and in yeast and other fungal cells [3]. The acyl-lipid desaturases introduce double bonds into fatty acids that have been esterified to glycerolipids [4–6]; they are bound to the endoplasmic reticulum, the chloroplast membranes in plant cells [5,7] and the thylakoid membranes in cyanobacterial cells [8].

We previously isolated the *desA*, *desB* and *desC* genes for Δ12, ω3 and Δ9 acyl-lipid desaturases from the cyanobacterium *Synechocystis* sp. PCC 6803 [9–11], and Reddy et al. [12] isolated the *desD* gene for Δ6 acyl-lipid desaturase from the same cyanobacterium. We compared the amino acid sequences of the four acyl-lipid desaturases and found eight conserved histidine residues (Fig. 1). These conserved histidines are found within the three conserved domains of histidine clusters [1]. It has been demonstrated that such conserved histidine residues are involved in the catalytic actions of many enzymes, for example dehydrogenases [13], and can bind metal irons [14]. Histidine, with its weakly basic imidazole group (pK_a = 6.0), is the amino acid that can donate a proton to serve as a ligand to transition metals in the neutral pH range.

In order to examine the importance of the histidine residues in the acyl-lipid desaturases we used site-directed mutagenesis to replace H90, H129, H287 and H290 in the conserved domains and one other histidine residue, H109, which is conserved only in the Δ12 and ω3 desaturases [9,15], by arginine, an amino acid that contains a strongly basic guanidium group (pK_a = 12.5). Then we analyzed the effects of the mutations on the activity of the enzyme.

2. Materials and methods

2.1. Site-directed mutagenesis

A 1.0-kb fragment of the *desA* gene of *Synechocystis* sp. PCC 6803, which contained the open-reading frame but not the promoter region, was subcloned into the expression vector pET-3a to generate the plasmid pET-desA, as previously described [16]. Specific base changes were introduced directly into pET-desA by the method of Deng and Nickoloff [17], using the Transformer TM Site-Directed Mutagenesis Kit ver. 2.0 (Clontech Laboratories, Palo Alto, CA, USA). Two oligonucleotides were simultaneously annealed to one strand of the denatured double-stranded pET-desA. One primer (referred to the mutagenic primer) was used to introduce the desired mutation and the other primer (the selection primer) was used to mutagenize a unique restriction site in the plasmid for subsequent selection. Amplification of the mutated strand, as well as of the parent strand, was achieved in a repair-deficient strain of *E. coli*, namely, BMH71-18 *muts* (Takara, Kyoto, Japan). After digestion of the parent plasmids with the selection-specific restriction enzyme, the mutagenized plasmids were amplified and cloned in *E. coli* HB101 (Takara). All positions of nucleotides and amino acids refer to those in the published sequence of the *desA* gene and its product [11]. Oligonucleotides were synthesized by the phosphoramidite method [18] with a DNA synthesizer (391 PCR Mate; Applied Biosystems, Tokyo, Japan). The nucleotide sequences of the mutagenic primers used for mutagenesis were: 5′-CTTCGTTGTCG-GCCGTGACTGTGGCCATCG-3′ (with an A to G sense-strand substitution at nucleotide 435) for substitution of histidine by arginine at position 90 (this mutation was designated H90R); 5′-TGATTTAGT-GGGACGTATCGCTTTTGCTCC-3′ (with an A to G sense-strand substitution at nucleotide 492) for substitution of histidine by arginine at position 109, (this mutation was designated H109R); 5′-ACTCCA-CGACCACCGTCACCTCCACACCAA-3′ (with an A to G sense-strand substitution at nucleotide 552) for substitution of histidine by arginine at position 129 (this mutation was designated H129R);

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Abbreviations: ACP, acyl carrier protein; IPTG, isopropyl-1-thio-β-D-galactoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid. Nomenclature for fatty acids: X:Y(Z), fatty acid containing X carbon atoms with Y double bonds in the *cis* configuration at position Z, counted from the carboxyl terminus.

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5′-TGACATCAACGTCCGTATTCCCCACCACC-3′ (with an A to G sense-strand substitution at nucleotide 1,026) for substitution of histidine by arginine at position 287 (this mutation was designated H287R); and 5′-CGTCCATATTCCCCGCCACCTCTCCGTTGC-3′ (with an A to G sense- strand substitution at nucleotide 1,035) for substitution of histidine by arginine at position 290 (this mutation was designated H290R).

All the mutations were verified by determination of the sequences of mutagenized regions by the dideoxy chain-termination method of Sanger et al. [19], using a sequencing kit (Sequenase ver. 2.0; USB, Cleveland, OH, USA).

2.2. *Expression of the* desA *gene and its derivatives in* E. coli

E. coli strain BL21(DE3) was transformed with pET-desA and with the various mutagenized plasmids by the procedures described by Hoey et al. [20]. Small single colonies of transformed *E. coli* cells were transferred to LB medium [21] that had been supplemented with 0.2 mg/ml ampicillin. The cells were grown for 8 h and cultures were stored at −80°C in the presence of 15% (w/w) glycerol.

For the expression of the *desA* gene, the frozen cells were streaked on LB plates supplemented with 1.5% agar and 0.2 mg/ml ampicillin. After incubation 37°C for 8 h, a single colony was transferred to 5 ml of M9 medium [21] supplemented with 0.2 mg/ml ampicillin, 4 mg/ml glucose, 0.5 μg/ml vitamin B1 and 1 mg/ml casamino acids, and the cells were grown at 37°C for 12 h. Then 4 ml of this preculture were inoculated into 200 ml of the above-mentioned M9 medium that had been further supplemented with 1 mM sodium oleate and 10 μM $FeCl_3$. The *E. coli* cells were grown at 37°C with shaking. When absorbance at 600 nm reached 0.5, IPTG at a final concentration of 0.4 mM was added and the culture was incubated for another hour. The cells were collected and then homogenized, as described previously [16], in a chilled French pressure cell (FA-030; SLM Instruments, Urbana, IL, USA) operated at 70 MPa. After removal of the unbroken cells by centrifugation at 1,800 × *g* at 4°C for 5 min, the supernatant, referred to herein as the homogenate, was immediately used for assays of desaturase activity and analysis of proteins.

2.3. *Characterization of expressed proteins*

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described by Laemmli [22] on a 12% polyacrylamide gel. Concentrations of protein were determined by the method of Bradford [23] with bovine serum albumin as the standard. The amino-terminal sequence of the *desA* product was determined by the method of Legendre and Matsudaira [24].

2.4. *Assay of desaturase activity*

Aliquots of homogenate corresponding to 300 μg of protein were assayed as described previously [16] with ferredoxin from spinach (Sigma, St. Louis, MO, USA), ferredoxin:$NADP^+$ oxidoreductase from spinach (Sigma) and NADPH (Sigma) as cofactors. Each reaction was allowed to proceed for 10 min at 25°C. Then total lipids were immediately extracted and subjected to methylation in 5% HCl in CH_3OH (w/v) for 2.5 h at 85°C [25]. An aliquot of 15:0 was added to each sample before methylation as an internal standard. Fatty acid methyl esters were analyzed by gas-liquid chromatography as previously described [16]. The amount of 18:2(9,12) was determined by comparing the peak area of 18:2(9,12) with that of 15:0 on the chromatogram.

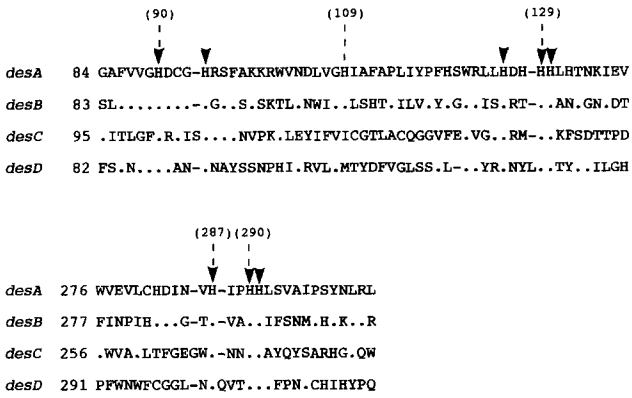

Fig. 1. Comparison of the sequences of four acyl-lipid desaturases from *Synechocystis* sp. PCC 6803. The regions containing the conserved amino-acid residues that were mutagenized in this study are shown. The sequences are those of the Δ12 desaturase (*desA*) [11], ω3 desaturase (*desB*) [9], Δ9 desaturase (*desC*) [9] and Δ6 desaturase (*desD*) [12]. The single-letter amino-acid code is used. Numbers correspond to the number of the first amino acid in that region, where the initial methionine of the protein is taken as number 1. Dots indicate amino-acid identity with Δ12 desaturase, and dashes indicate gaps that were introduced to optimize the alignment. Arrowheads indicate eight histidine residues that are conserved among the four desaturases. Numbers in parentheses indicate the histidine residues that were mutagenized in this study.

3. Results and discussion

3.1. *Expression of the desA gene in* E. coli

Table 1 shows a comparison of levels of fatty acids in homogenates of *E. coli* cells that had been transformed with pET-3a and pET-desA after incubation for 10 min in the assay mixture. The high level of 18:1(9) in all the samples was due to its presence in the original culture medium. In the cells that had been transformed with pET-desA, the level of 18:2(9,12) increased at the expense of 18:1(9) but levels of the other fatty acids did not change significantly during the incubation in the assay mixture. By contrast, the levels of all fatty acids remained constant during incubation with a homogenate prepared from cells that had been transformed with pET-3a. These results demonstrate that the homogenate of *E. coli* cells that had been transformed with pET-desA exhibited high activity of Δ12 desaturase in vitro.

Table 1
Changes in the levels of fatty acids in homogenates prepared from *E. coli* cells after transformation with pET-3a or pET-desA

Strain	Incubation time (min)	Fatty acid					
		16:0	16:1 (9)	18:0	18:1 (9)	18:1 (11)	18:1 (9, 12)
		(nmol/mg protein)					
BL21 (DE3)/pET-3a	0	113 ± 21	19 ± 3	10 ± 4	241 ± 36	47 ± 8	0
	10	113 ± 19	18 ± 3	10 ± 4	255 ± 42	50 ± 8	0
BL21 (DE3)/pET-desA	0	125 ± 24	21 ± 4	14 ± 3	300 ± 60	56 ± 15	11 ± 3
	10	133 ± 23	22 ± 6	13 ± 3	250 ± 65	64 ± 17	51 ± 21

E. coli cells were cultured in the presence of 1 mM sodium oleate. A homogenate of cells was incubated in the assay mixture, as described in section 2. Lipids were extracted, methylated and analyzed by gas-liquid chromatography. The results are the means of results from at least three independent experiments.

As observed previously [16], a significant amount of 18:2(9,12) was detected prior to incubation in the homogenate of *E. coli* cells that had been transformed with pET-desA (Table 1). However, this fatty acid was totally absent in the case of the control strain of *E. coli* that had been transformed with pET-3a. These results indicate that electrons for the desaturation are donated to the Δ12 desaturase from some cofactor(s), such as ferredoxin, in *E. coli* cells [26], which allows the desaturation of 18:1(9) to proceed. This activity is referred to as activity in vivo, to distinguish it from the activity in vitro.

3.2. Site-directed mutagenesis of Δ12 desaturase

The cellular proteins in the homogenates from the variously transformed *E. coli* cells were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). A protein with a molecular mass similar to that of the product of the unmodified *desA* gene, namely, a 36-kDa polypeptide, was synthesized at similar levels in all cases. To confirm that the 36-kDa polypeptide was the product of the *desA* gene, we determined the amino-terminal sequence of each 36-kDa polypeptide. The amino-terminal sequence was Ala-Arg-Thr-Pro-Thr-Val-Thr-Pro-Ser-Asn-. This result confirmed that each 36-kDa polypeptide was the product of the *desA* gene, which had been modified in the construction of pET-desA such that the first 8 amino acids were replaced by Met-Ala-Arg-. The absence of the first methionine in the determined sequence suggests that it was removed by the post-translational machinery of *E. coli* [27].

Table 2 shows effects of mutations of the histidine residues on the activities in vivo and in vitro of the Δ12 desaturase synthesized in *E. coli* cells. The mutations at the conserved sites, H90, H129, H287 and H290, totally eliminated the activity in vitro, and has a considerable negative effect on the activity in vivo. These findings suggest that the conserved histidine residues H90, H129, H287 and H290 are all essential for the activity of the Δ12 desaturase. The presence of low levels of activity in vivo cannot be well explained at present. The separate mutation of H109 completely eliminated the activities both in vivo and in vitro. It is noteworthy that this latter histidine residue is conserved in the Δ12 acyl-lipid desaturases of cyanobacterial strains such as *Synechocystis* sp. PCC 6714, *Synechococcus* sp. PCC7002 and *Anabaena variabilis* [15], and also in those of higher plants such as *Arabidopsis thaliana* [28], rapeseed [29], soybean [29] and spinach [30], as well as in the ω3 acyl-lipid desaturases of *Synechocystis* sp. PCC6803 [9], *A. thaliana* [31–33], rapeseed [33], soybean [33] and mung bean [34]. However, this histidine residue is not conserved in the Δ9 and Δ6 desaturases of *Synechocystis* sp. PCC 6803. Although H109 is not conserved in all the desaturases, it appears that it is essential for Δ12 desaturation.

Table 2
Effects of mutations of the conserved histidine residues on the activities in vivo and in vitro of Δ12 desaturase

Mutation	Before incubation (activity in vivo)	After incubation	Difference (activity in vitro)
	(nmol 18:2 (9,12)/mg protein)		
Control (pET-*desA*)	11 ± 3	51 ± 20	40 ± 23
H90R	3.2 ± 1.0	2.8 ± 1.0	0
H109R*	0.0 ± 0.5	0.0 ± 0.5	0
H129R	1.6 ± 0.3	1.1 ± 0.5	0
H287R	1.9 ± 0.5	1.5 ± 0.5	0
H290R	1.5 ± 1.0	1.4 ± 1.0	0

*H109 is conserved in the Δ12 and ω3 desaturases, but not in the Δ9 and Δ6 desaturases. The desaturase activity of a homogenate of *E. coli* cells was measured as described in the footnote to Table 1. The results are means of results from two or three independent experiments.

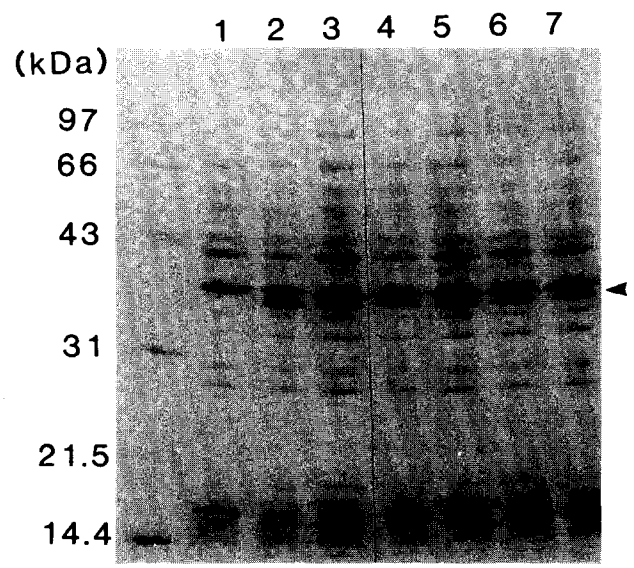

Fig. 2. Expression of wild-type and mutagenized *desA* genes in *E. coli*. Aliquots (0.6 ml) of cultures after induction by IPTG were harvested and the total cellular proteins were solubilized and subjected to electrophoresis on an SDS-polyacrylamide (12%) gel. Molecular mass markers are shown on the left. Lane 1, control cells that had been transformed with pET-3a; lane 2, cells that had been transformed with pET-desA. Lanes 3 to 7 represent cells that had been transformed with plasmids that included mutagenized *desA* genes. Lane 3, H90R mutation; lane 4, H109R mutation; lane 5, H129R mutation; lane 6, H287R mutation; lane 7, H290R mutation. The arrowhead indicates the position of the product of the *desA* gene.

Shanklin et al. [35] mutagenized eight conserved histidine residues in the stearoyl-CoA desaturase of the rat, converting them to alanine residues which are hydrophobic and electrically neutral, and they found that these mutations eliminated the ability to sustain growth of a yeast mutant that was defective in stearoyl-CoA desaturase. Although their data were only qualitative, they proposed that the conserved histidine residues are essential for the activity of stearoyl-CoA desaturase. This proposal is consistent with our conclusion related to the importance of the histidine residues in the acyl-lipid desaturases.

To obtain further information about the characteristics of the various mutant acyl-lipid desaturases, such as their iron content and the impact of each mutation on the physical and biochemical features of the enzyme, the next and crucial step is the purification, without loss of activity, of the acyl-lipid desaturases from *E. coli* cells that have been transformed with the genes for the desaturases. Efforts in this direction are in progress.

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