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Structure–function studies of yeast C-4 sphingolipid long chain base hydroxylase

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Abstract

The roles of putative active site residues of the *Saccharomyces cerevisiae* sphingolipid C-4 long chain base hydroxylase (Sur2p) were investigated by site-directed mutagenesis. The replacement of any one of conserved His residues of three histidine-rich motifs with an alanine eliminated hydroxylase activity in vivo and in vitro, indicating that they are all essential elements of the active site. An additional conserved His residue (His 249) outside of the histidine-rich cluster region was also found to be crucial for activity. Additional mutants altered in residues in close proximity to the histidine-rich cluster were generated. In order to determine their roles in hydroxylase vs. desaturase activities, residues were replaced with conserved residues from the yeast Δ7-sterol-C5(6)-desaturase, Erg3p. Residues Phe 174, Asn 182, Ser 191, Leu 196, Pro 199, Asn 266, Tyr 269, Asp 271 and Gln 275 appear to be additionally important elements of the active site but their conversion into corresponding Erg3p residues did not lead to a gain in desaturase activity. It is concluded that Sur2p is a membrane-bound hydroxylase that belongs to the diiron family of eight-histidine motif enzymes.

Keywords: Sphingolipid; *Saccharomyces cerevisiae*; C-4 long chain base hydroxylase; *SUR2*; Site-directed mutagenesis

1. Introduction

Sphingolipids are particularly important and abundant molecules with structural and regulatory functions [1,2]. For example, ceramide, the base compound of sphingolipids, and sphingosine-1-phosphate are important signaling molecules. They are generated in response to a variety of stimuli, such as cytokines, UV light, heat, DNA damage, growth factors, G protein-coupled receptor agonists and antigens. The generation of ceramide indicates cellular stress and has been implicated in apoptosis, proliferation and differentiation [3]. Sphingosine-1-phosphate is an extracellular effector involved in apoptosis, differentiation and migration, and it also functions as an intracellular second messenger [4].

Sphingolipids are composed of a long chain base with an amide-linked fatty acid at the C-2 position and a polar constituent at the C-1 position. There are significant differences between sphingolipids of mammals and the yeast *Saccharomyces cerevisiae*. Yeast produces primarily the long chain base known as phytosphingosine (PHS), which is hydroxylated at the C-4 position. In mammals, the long chain bases are mostly in the form of sphingenine, desaturated at the C4(5) position. C-4 hydroxylated sphingolipids also occur in small amounts in mammalian cells in skin, kidney, intestine and erythrocytes [5–7]. The amount of hydroxylated sphingolipids is significantly high in human carcinomas. For instance, human colonic and liver adenocarcinomas possess sphingolipids with hydroxylated sphingoid bases and fatty acids [8]. The presence of these hydroxylated sphingolipids could change cell surface properties that lead to cancer. They may also alter ceramide-induced apoptosis [9], a phenomenon in which the accumulation of C4(5) desaturated ceramide is often observed [10]. In carcinomas, a possible limitation of this desaturation and a reciprocal increase in C-4 hydroxylation prevent normal ceramide-induced apoptosis.

Despite a great deal of research on the involvement of hydroxylated sphingolipids in cancer, only one report reveals a low level of C-4 hydroxylase activity [11].

On the other hand, the gene responsible for sphingoid base C-4 hydroxylation in *S. cerevisiae* has been identified and studied. It was first recognized as gene *SUR2* (*SYR2*), necessary for the antifungal action of syringomycin E, a

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small lipodepsinonapeptide secreted by *Pseudomonas syringae* [12,13]. Later, it was recognized to encode an enzyme required for the long chain base hydroxylation in yeast [12,14]. Sur2p catalyzes hydroxylation at the C-4 position at the level of dihydrosphingosine (Fig. 1). *SUR2* homologs have been identified in *Arabidopsis thaliana* [15], *Schizosaccharomyces pombe* (accession number: CAA21900) and *Candida albicans* (ORF sequence available from http://www-sequence.stanford.edu).

Multiple sequence alignment of Sur2p homologs from *C. albicans*, *A. thaliana*, *S. pombe* and *S. cerevisiae* shows that all are identical in eight-conserved histidine residues, which form three conserved clusters (Fig. 2). Two histidine-containing motifs are located in the loop between the predicted second and third transmembrane segment. The third histidine-rich motif follows the fourth predicted transmembrane segment.

Shanklin et al. [16] catalogued 75 known proteins that contain similar eight-histidine motifs. The histidines are proposed to bind catalytically active diiron. These proteins are integral membrane proteins with desaturase, hydroxylase, oxidase or decarbonylase activities. The tripartite histidine motif is thought to be an important part of the active site of these enzymes, where it is proposed to bind catalytically active diiron. Spectroscopic studies of alkane ω-hydroxylase from *Pseudomonas oleovorans* [16]—an enzyme of this family—indicate the presence of a diiron cluster. The role of highly conserved histidines in enzymatic catalysis is indicated by site-directed mutagenesis studies of family members with desaturase functions: the Δ7-sterol-C5(6)-desaturase from *A. thaliana* [17], the Δ12 acyl-lipid desaturase of *Synechocystis* [18] and the rat Δ9 fatty acid desaturase [19]. In contrast, structure–function studies on the role of the histidine residues for hydroxylase activity have not yet been reported.

In this work, site-directed mutagenesis studies of Sur2p with systematic alteration of each histidine residue and neighboring amino acids were undertaken. In addition, Sur2p residues were replaced with corresponding residues from a closely related yeast desaturase, Erg3p [20]. The studies not only shed light on the roles of the histidines in hydroxylase action of Sur2p, but also on the specific contributions of other amino acids to hydroxylase vs. desaturase activities.

2. Materials and methods

2.1. Site-directed mutagenesis

The pYES2 yeast expression vector (Invitrogen, Carlsbad), initially containing the wild-type *SUR2* gene cloned into *Pst*I–*Sph*I restriction sites [13], was used as a template. For multiple site mutations, previously mutagenized plasmids were used as templates. Site-directed mutagenesis was performed using a method previously described [21], with some modifications. Two mutagenized primers were used to prime DNA synthesis by a high–fidelity polymer-

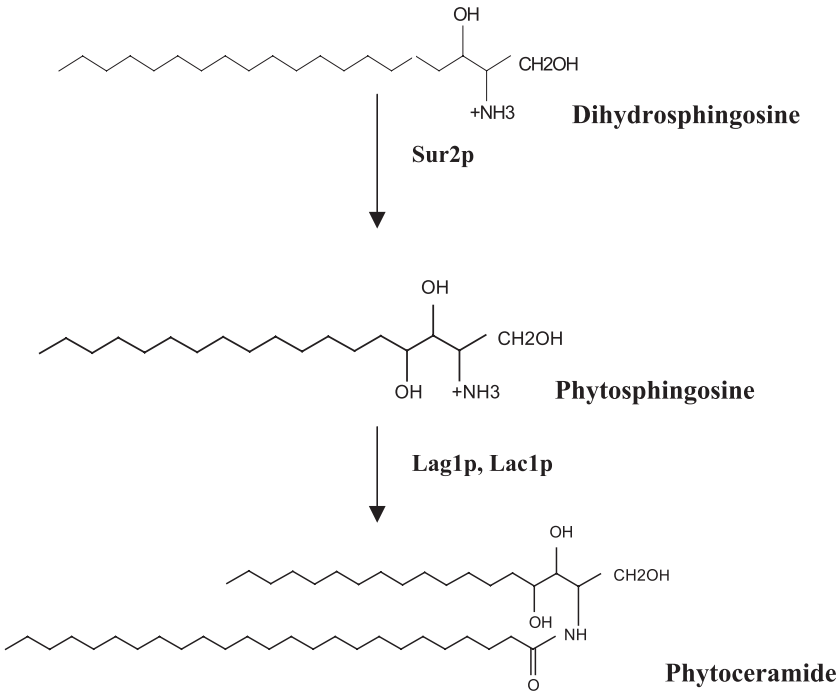

Fig. 1. Biosynthesis of ceramide in yeast. The following enzymes are indicated: Sur2p—C-4 sphingoid base hydroxylase, Lag1p, Lac1p—ceramide syntheses.

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Sur2p          MNVTSNATAAGSFPLAFGLKTSFGFMHYAKAPAINLRPKESLLPEMSDGVLALVAPVVAY 60
C. albicans    ---MSTFKPPANFSRVLSQPTFY--------EKIIITEQPSLIKGIPDGILALIVPVVAY
S. pombe       ----------------------------------MVTTVEMLTTWNPVTVSLVSPVIIY
A.thaliana 1   -------------------------------------MMSFVISDEFLGTFVPILVY
A.thaliana 2   ----------------------------------------MGFISGEELAVTLAPVAVY
Erg3p          -MDLVLEVADHYVLDDLYAKVLPASLAANIPVKWQKLLGLNSGFSNSTILQETLNSKNAV

Sur2p          WALSGIFHVIDTFHL--AEKYR-IHPSEEVAKRNKASRMHVFLEVILQHIIQTIVGLIFM 117
C. albicans    WSYSMFFHIIDVYEL--AEQYR-IHPSEEEKSRNKVTLHEVVRDVIFQHIIQTIAGFAVY
S. pombe       WVASAFFGFLHYIELPVFEKYR-IHPPEEIARRNRVPQMAVVKAVLFQQLCEVVVGIALA
A.thaliana 1   WVYSGMY--ICLG---SLDKYR-LHSKIDEDEKNLVSKSAVVKGVLLQQTLQAIISVILF
A.thaliana 2   WLYSGIY--EALGSVRALDRYR-LHSRRDEESNNMASKKEVVKGVLLQQAIQVAISLAVL
Erg3p          KECRRFYGQVPFLFDMSTTSFASLLPRSSILREFLSLWVIVTIFGLLLYLFTASLSYVFV

Sur2p          HFEPIYMTGFEENAMWKLRAD--------------------LPRIIP--DAAIYYGYMYG 155
C. albicans    CIDPIPKTGYELYTMWNLKYNY-------------------LPSFVP--DWAIYYGYMYG
S. pombe       MFEGYPEPIDEAKQMLRYEAFFSK----------------NLPALLQVAPFAPKLAYNFI
A.thaliana 1   KITG-----------------------------------------SDADAATTQQFSIL
A.thaliana 2   KLTSEKDGGAGDVKAGHASAP-----------------------AAAPSSSSAAAALL
Erg3p          FDKSIFNHPRYLKNQMAMEIKLAVSAIPWMSMLTVPWFVMELNGHSKLYMKIDYENHGVR

Sur2p          MSALKIFAGFLFVDTWQYFLHRLMHMNKTLYKWFHSVHHELYVPYAYGALFNNPVEGFLL 215
C. albicans    WSFLRILIAFCIIDSWQFWLHRLMHINKSLYRRFHSRHHRLYVPYAFGALYNDPVEGFLL
S. pombe       VPAFQYFFAFFIIDSWQYFWHRYLHYNKKLYNMIHAHHRLQVPYAMGALYNHPFEGLIL
A.thaliana 1   LLARQFIIAMLVIDTWQYFIHRYMHLNKFLYKHIHSQHHRLIVPYSYGALYNHPLEGLLL
A.thaliana 2   DVAARFGVAMFVLDAWQYFAHRLMHSSRYMYRRFHSWHHRVVAPYAFAAQYGHPVDGVLT
Erg3p          KLIIEYFTFIFFTDCGVYLAHRWLHWPR-VYRALHKPHHKWLVCTPFASHSFHPVDGFLQ

Sur2p          DTLGTGIAMTLTHLTHREQIILFTFATMKTVDDHCGYALPLDPFQWLFPNNAVYHDIHHQ 275
C. albicans    DTLGTGIASLVTGLSHRESIFLYTFATLKTVDDHCGYRLPFDIFQIIFPNNSVYHDIHHQ
S. pombe       DTFGAGVAYLAAGLSPQQAVIFFTLSTLKTVDDHCGYVFPYDPLQMFFANNARYHDLHHQ
A.thaliana 1   DTIGGALSFLFSGMSPRTAIFFFSFATIKTVDDHCGLWLPGNPFHIFFSNNSAYHDVHHQ
A.thaliana 2   EALSGAAAYLASGLPPRAAAFFLAFATVKGIDDHCGLLVPWNPLHAAFANNTAYHDVHHQ
Erg3p          S-ISYHIYPLILPLHKVSYLILFTFVNFWTVMIHDGQYLSNNP----AVNGTACHTVHHL

Sur2p          QFGIKTNFAQPFFTFWDNLFQTNFKGFEEYQKKQRRVTIDKYKEFLQERELEK---KEKL 332
C. albicans    MWGIKNNFSQPFFTFWDVLNNTQYKFVNEYKDLQKHITLTKYKEFLAKKSGSKSKSKQEL
S. pombe       PYGFQKNFSQPFFTFWDHVLGT-------------------YMPPKSETPYEK---KQKA
A.thaliana 1   LYGTKYNFSQPFFVMWDRILGTYLP----------------YSLEKRAN---GGFETRPI
A.thaliana 2   LSGGRRNFSQPFFVVWDRLLGTHAG----------------YTVTARERNNGGGLEAKPI
Erg3p          YFN--YNYGQ-FTTLWDRLGGSYRRP----DDSLFDPKLRDAKETWDAQVKEVEHFIKEV

Sur2p          KNFKAMNAAENEVKKEK-- 349
C. albicans    EIEEEEKSIDKETKKEI--
S. pombe       KNAKKVN------------
A.thaliana 1   KVSKDE-------------
A.thaliana 2   CNDRHVLL-----------
Erg3p          EGDDNDRIYENDPNTKKNN
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Fig. 2. Alignment of Sur2p protein with sphingoid base hydroxylases from different species and with *S. cerevisiae* CΔ7-sterol-C5(6)-desaturase (Erg3p). Alignment was obtained by Clustal W analysis [35]. Residues altered in this study are in boldface. Conserved histidines are underlined.

ase (Platinum Taq, Invitrogen). A denaturated plasmid served as template. Primers used are shown in Table 1. The amplification was performed using an Ericomp PowerBlockI™/PowerBlockII™ PCR System. A two-stage amplification reaction was used [22]. In the first step, two separate amplifications—each containing one of the two oligonucleotides—were performed using the following conditions: one cycle of 30 s at 94 °C, followed by five cycles of 30 s at 94 °C, 1 min at 55 °C and 7.5 min at 68 °C with final elongation of 10 min at 68 °C. Equal volumes of both reactions were mixed and second stage amplification was performed with PCR cycling parameters: 1 cycle of 30 s at 94 °C, followed by 15 cycles of 30 s at 94 °C, 1 min at 55 °C and 7.5 min at 68 °C with final elongation of 10 min at 68 °C. Products of amplifications were digested with *Dpn*I restriction enzyme in order to eliminate the methylated parental template molecules. After chloroform extraction and ethanol precipitation about 10 μg of DNA were used to transform competent *Escherichia coli* TG1 cells (supE thi-1 D(lac-proAB) D(mcrB-hsdSM)5 (rK- mK-) [*F′* traD36 proAB lacIqZDM15]). Transformants were selected for antibiotic resistance on medium containing 100 μg/ml ampicillin. Plasmid DNAs extracted from *E. coli* transformants were sequenced to verify that the desired mutations were introduced and no additional mutations were present in the *SUR2* coding region.

2.2. Yeast transformation

Competent cells were prepared as described [23] and yeast was transformed by electroporation. DNA containing a mutated *SUR2* gene was used to transform the BY4742 *sur2*Δ (MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *sur2*Δ::*KAN*)

Table 1
Synthetic oligonucleotide primers used for site-directed mutagenesis (codons for changed amino acids are underlined and product of the point mutation within the codons for changed amino acids are in boldface)

Phe 174 Leu	CATGGCAATAC**CTT**TTGCATAGATTG
	CAATCTATGCAA**AAG**GTATTGCCATG
His 176 Ala	GGCAATACTTTTTG**GCT**AGATTGATGCATATG
	CATATGCATCAATCT**AGC**CAAAAAGTATTGCC
His 180 Ala	GCATAGATTGATG**GCT**ATGAATAAGACC
	GGTCTTATTCAT**AGC**CATCAATCTATGC
Asn 182 Pro	GATGCATATG**CCT**AAGACCTTATAC
	GTATAAGGTCTT**AGG**CATATGCATC
His 190 Ala	CCTTATACAAATGGTTC**GCC**TCTGTTCATCATG
	CATGATGAACAGA**GGC**GAACCATTTGTATAAGG
Ser 191 Lys	CAAATGGTTCAAC**AAG**GTTCATCATGAAC
	GTTCATGATGAAC**CTT**GTGGAACCATTTG
His 193 Ala	GGTTCCACTCTGTT**GCT**CATGAACTATACGTGC
	GCACGTATAGTTCATG**AGC**AACAGAGTGGAACC
His 194 Ala	CCACTCTGTTCAT**GCT**GAACTATACGTGC
	GCACGTATAGTTC**AGC**ATGAACAGAGTGG
Leu 196 Trp	GTTCATCATGAA**TGG**TACGTGCCATATGC
	GCATATGGCACGTA**CCA**TTCATGATGAAC
Pro 199 Cys	GAACTATACGTG**TGC**TATGCTTACGG
	CCGTAAGCATA**GCA**CACGTATAGTTC
His 249 Ala	GACTGTCGATGAC**GCC**TGTGGGTATGC
	GCATACCCACA**GGC**GTCATCGACAGTC
Asn 266 Gly	GCTTTTCCCTAAT**GGC**GCTGTCTATCACG
	CGTGATAGACAGC**GCC**ATTAGGGAAAAGC
Tyr 269 Cys	CCTAATAACGCTGTC**TGT**CACGATATCC
	GGATATCGTGACAG**ACA**GCGTTATTAGG
His 270 Ala	CCTAATAACGCTGTCTAT**GCC**GATATCCACCACC
	GGTGGTGGATATC**GGC**ATAGACAGCGTTATTAGG
Asp 271 Thr	GCTGTCTATCAC**ACT**ATCCACCACC
	GGTGGTGGAT**AGT**GTGATAGACAGC
His 273 Ala	GCTGTCTATCACGATATC**GCC**CACCAGCAATTTGG
	CCAAATTGCTGGTG**GGC**GATATCGTGATAGACAGC
His 274 Ala	CACGATATCCAC**GCC**CAGCAATTTGG
	CCAAATTGCTG**GGC**GTGGATATCGTG
Gln 275 Leu	CGATATCCACCAC**CTG**CAATTTGGTATC
	GATACCAAATTG**CAG**GTGGTGGATATCG

strain (Research Genetics). BY4742 *sur2*Δ:*SUR2* strain was used as a positive control.

2.3. Growth conditions

Yeast was grown at 28–30 °C with shaking in minimal uracil dropout medium containing glucose [24]. In order to induce the gene under control of the *GAL1* promoter, cells were transferred to minimal uracil dropout medium containing galactose (2%) as a carbon source.

2.4. Long chain base analysis

Yeast was grown as described above and harvested by centrifugation. Release and identification of long chain bases were performed as previously described [25]. Briefly, lipids of the cell pellet were hydrolyzed by adding 1 M HCl in methanol/water (82:18, v/v) and incubating at 80 °C for 20 h. After heating, ammonium hydroxide was added, then sphingoid bases were extracted with chloroform and dried. Next, derivatization with UV-absorbing 4-biphenylcarbonylchloride (Sigma) was carried out and samples were resolved by reverse phase HPLC on a 4.6 mm × 15 cm Beckman C-18 column. Long chain bases were eluted with the following gradient profile: 85% methanol, 5 min, 85–95% methanol linearly, 20 min, 95% methanol, 5 min, 95–85% methanol linearly, 5 min and 85% methanol, 5 min. The effluent was monitored by absorbance at 280 nm.

2.5. C-4 hydroxylase assay

The assay was performed as described by Grilley and Takemoto [26]. Mutants were grown on medium lacking uracil and induced with galactose. Cells were harvested, spheroplasted using Zymolase 100T (Seikagaku Corp.) and disrupted by sonication. Finally, microsomal membranes were isolated by differential centrifugation. For the in vitro enzyme reaction, sphingoid base substrate dihydrosphingosine (DHS) (Avanti Polar-Lipids, Inc.) was mixed with the microsomes and NADPH to initiate the reaction. The reaction mixture was incubated at 25 °C for 1 h and stopped with methanol/chloroform (2:1, v/v). Next, the reaction solution was centrifuged to separate the chloroform phase that contains sphingoid bases. The chloroform phase was dried, derivatized with UV-absorbing 4-biphenylcarbonylchloride and subjected to high-performance liquid chromatography as described above. PHS (Avanti Polar-Lipids) derivatized with biphenylcarbonylchloride was used as a standard. Control reactions using heat-inactivated (55 °C for 10 min) microsomes and without adding NADPH were run for BY4742 *sur2*Δ:*SUR2* to account for endogenous PHS. No detectable PHS was present in these reactions.

2.6. C4(5) desaturase assay

The assay was performed in the same way as the C-4 hydroxylase assay (above), except that the production of C4(5) desaturated sphingoid bases was analyzed. Sphingosine (Avanti Polar-Lipids) derivatized with biphenylcarbonylchloride was used as a standard.

2.7. Syringomycin E treatment

Cells were grown on plates containing minimal uracil dropout medium with glucose and replica plated on uracil dropout medium with glucose or galactose containing 1 μg/ml of syringomycin E. Growth was examined after 36 h incubation at 28–30 °C.

2.8. SDS-PAGE and immunoblotting

Microsome preparations (50 μg of protein per lane) were analyzed by 10% SDS-PAGE. After electrophoresis, proteins were electrotransferred onto PVDF membranes at 15 V for 18 h. Transferred proteins were detected by Western blotting as previously described [27]. Duplicate gels were

run and stained with Coomassie blue to estimate relative total protein loaded in each lane.

3. Results

3.1. Mutation of His residues

Site-directed mutagenesis of each of the eight conserved His residues of Sur2p was done to determine their roles in sphingoid base hydroxylation. Mutants of residues 176, 180, 190, 193, 194, 270, 273 and 274 from the three histidine-rich motifs were constructed. The residues were individually replaced with alanine. Sur2p levels were examined in all mutants by Western blot analysis, and they were similar to the wild-type levels.

The replacement of each of the conserved His residues from the three histidine-rich motifs completely eliminated hydroxylase activity of Sur2p. No phytosphingosine was present in sphingolipids extracted from mutants (Table 2). In addition, in vitro activity assays did not reveal C-4 hydroxylase activities in microsomal preparations from the His mutants (Table 3).

3.2. Mutation of His 249

An additional conserved His residue (His 249) outside of the histidine-rich cluster region was studied. Replacement of His 249 by an alanine residue resulted in complete loss of hydroxylase activity both in vivo and in vitro (Tables 2 and 3). This indicates that the additional histidine, located between the second and third cluster of highly conserved histidines is crucial to the reaction catalyzed by Sur2p.

Table 2
Sphingoid base analysis

	PHS (%)
BY4742 *sur2*Δ	< 0.09
BY4742 *sur2*Δ:*SUR2*	41.46 ± 2.56
BY4742 *sur2*Δ:*SUR2* His 176 Ala	< 0.23
BY4742 *sur2*Δ:*SUR2* His 180 Ala	< 0.15
BY4742 *sur2*Δ:*SUR2* His 190 Ala	< 0.08
BY4742 *sur2*Δ:*SUR2* His 193 Ala	< 0.12
BY4742 *sur2*Δ:*SUR2* His 194 Ala	< 0.1
BY4742 *sur2*Δ:*SUR2* His 270 Ala	< 0.08
BY4742 *sur2*Δ:*SUR2* His 273 Ala	< 0.11
BY4742 *sur2*Δ:*SUR2* His 274 Ala	< 0.09
BY4742 *sur2*Δ:*SUR2* His 249 Ala	< 0.08
BY4742 *sur2*Δ:*SUR2* Phe 174 Leu	< 0.11
BY4742 *sur2*Δ:*SUR2* Asn 182 Pro	< 0.18
BY4742 *sur2*Δ:*SUR2* Ser 191 Lys	53.14 ± 4.54
BY4742 *sur2*Δ:*SUR2* Leu 196 Trp	< 0.13
BY4742 *sur2*Δ:*SUR2* Pro 199 Cys	< 0.1
BY4742 *sur2*Δ:*SUR2* Asn 266 Gly	< 0.13
BY4742 *sur2*Δ:*SUR2* Tyr 269 Cys	< 0.1
BY4742 *sur2*Δ:*SUR2* Asp 271 Thr	49.84 ± 7.02
BY4742 *sur2*Δ:*SUR2* Gln 275 Leu	< 0.01

Percentage of PHS synthesized in yeast expressing wild-type and mutated forms of *SUR2*. 100%—total sphingoid bases.

Table 3
C-4 sphingoid base hydroxylase activity in *SYR2* null mutant and transformants carrying the mutagenized forms of Sur2p (relative to wild-type Sur2p)

	Hydroxylase activity (%)
Sur2p	100%
*sur2*Δ	not detected[a]
His 176 Ala	not detected
His 180 Ala	not detected
His 190 Ala	not detected
His 193 Ala	not detected
His 194 Ala	not detected
His 270 Ala	not detected
His 273 Ala	not detected
His 274 Ala	not detected
His 249 Ala	not detected
Phe 174 Leu	not detected
Asn 182 Pro	not detected
Ser 191 Lys	98.7 ± 7.09
Leu 196 Trp	not detected
Pro 199 Cys	not detected
Asn 266 Gly	not detected
Tyr 269 Cys	not detected
Asp 271 Thr	98.3 ± 6.08
Gln 275 Leu	not detected

[a] Less than 0.5%, when 100% equals the formation of 2.73 ± 0.71 nmol of PHS by wild-type form of Sur2p as a result of the in vitro assay with 500 μg of protein under conditions described in Materials and methods.

3.3. Mutation of Phe174Leu, Asn182Pro, Ser191Lys, Leu196Trp, Pro199Cys, Asn266Gly, Tyr269Cys, Asp271Thr and Gln275Leu

Additional conserved residues that are in close proximity to the histidine-rich clusters were studied to determine their roles in hydroxylase vs. desaturase activities. Broun et al. [28] showed that members of the diiron family of enzymes from plants are catalytically plastic and a few non-histidine amino acid substitutions near the histidine-rich motifs are capable of converting a fatty acid hydroxylase into a desaturase. There is no similarity between Syr2p and plant fatty acid enzymes studied by Broun et al., aside from the eight histidine residues characteristic of diiron family members. However, in order to examine similar potential changes in the catalytic activity of Sur2p, Sur2p was aligned with the yeast Δ7-sterol C5(6) desaturase Erg3p, the only known yeast desaturase with close similarity to Sur2p. Sequence alignment revealed a number of residues in the histidine-rich regions that are significantly different between Sur2p and Erg3p (Fig. 2). Mutants altered in these amino acids were constructed, and analyzed for hydroxylase activity. Sur2p levels were examined in all these mutants by Western blot analysis, and they were comparable to the wild-type. Hydroxylase activities were unchanged in Sur2p mutated at Ser 191 and Asp 271. All other mutations resulted in a loss of hydroxylase activity both in vivo and in vitro (Figs. 3 and 4; Tables 2 and 3). No concomitant gain of desaturase activity was evident. Taken together, residues Phe 174, Asn 182, Ser 191, Leu 196, Pro 199, Asn 266, Tyr

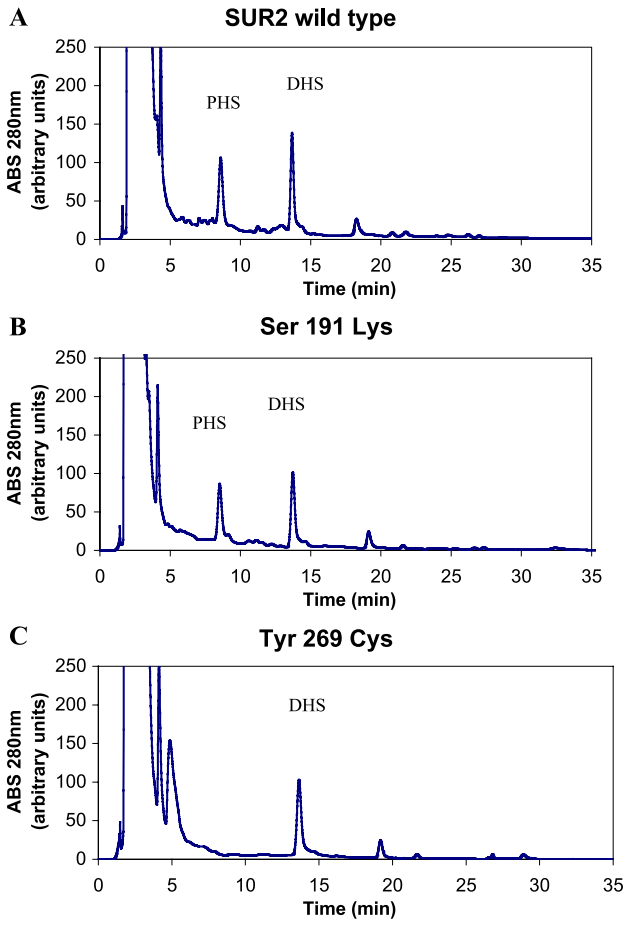

Fig. 3. Examples of sphingoid base analysis produced by wild-type Sur2p and mutated forms of the enzyme. Methanol–HCl hydrolysates of strain BY4742 *sur2*Δ expressing (A) wild-type form of *SUR2* (B) Ser 191 Lys, and (C) Tyr 269 Cys mutants, were derivatized with 4-biphenylcarbonyl chloride and the products were resolved by reverse phase HPLC as described in Materials and methods.

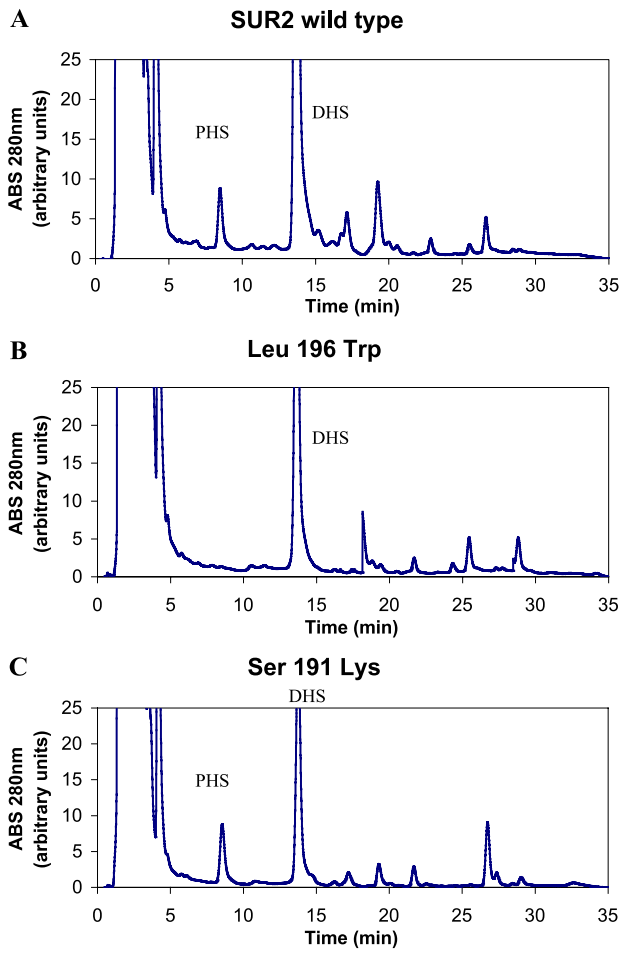

Fig. 4. Examples of in vitro hydroxylase assay using microsomal preparations from wild-type Sur2p and mutated forms of the enzyme. Microsomes from BY4742 *sur2*Δ expressing (A) wild-type form of *SUR2* (B) Leu 196 Trp, and (C) Ser 191 Lys mutants, were incubated with DHS substrate for 60 min at 25 °C, the sphingoid bases extracted with chloroform and derivatized with the 4-biphenylcarbonyl chloride, and the products were resolved by reverse phase HPLC as described in Materials and methods.

269, Asp 271 and Gln 275 appear to be additionally important elements of the active site of the yeast C-4 sphingoid base hydroxylase.

Since four or seven amino acid substitutions combined were required for the fatty acid hydroxylase–desaturase interconversion [28], multiple site mutations of Sur2p were also analyzed. Multiple site mutants were constructed in the combinations shown in Table 4. Neither desaturase nor hydroxylase activity was observed in these mutants. Interestingly, the level of Sur2p mutant protein synthesis was highly reduced in all cases (data not shown).

3.4. Syringomycin E sensitivity

All constructed mutants were tested for growth sensitivity to syringomycin E. Syringomycin E is a small lipodepsinonapeptide produced by the plant bacterium *P. syringae*, and it inhibits growth of *S. cerevisiae* at micromolar concentrations. The *SUR2* gene is required for the antifungal action of syringomycin E suggesting that C-4 sphingoid base hydroxylation of sphingolipids is necessary for syringomycin E action [13]. Only mutants altered in residues Ser 191 and Asp 271 were sensitive to this antifungal agent (Fig. 5) (data not shown for multiple site mutants), in accordance with the

Table 4
Multiple site mutants constructed in this study

Asn182Pro, Asn266Gly
Asn182Pro, Asn266Gly, Pro199Cys
Asn182Pro, Asn266Gly, Pro199Cys, Gln275Leu
Asn182Pro, Asn266Gly, Pro199Cys, Gln275Leu, Phe 174Leu
Asn182Pro, Asn266Gly, Pro199Cys, Gln275Leu, Phe174Leu, Tyr269Cys
Asn182Pro, Asn266Gly, Pro199Cys, Gln275Leu, Phe174Leu, Tyr269Cys, Leu196Trp
Asn182Pro, Asn266Gly, Pro199Cys, Gln275Leu, Phe174Leu, Tyr269Cys, Leu196Trp, Asp271Thr
Asn182Pro, Asn266Gly, Pro199Cys, Gln275Leu, Phe174Leu, Tyr269Cys, Leu196Trp, Asp271Thr, Ser191Lys

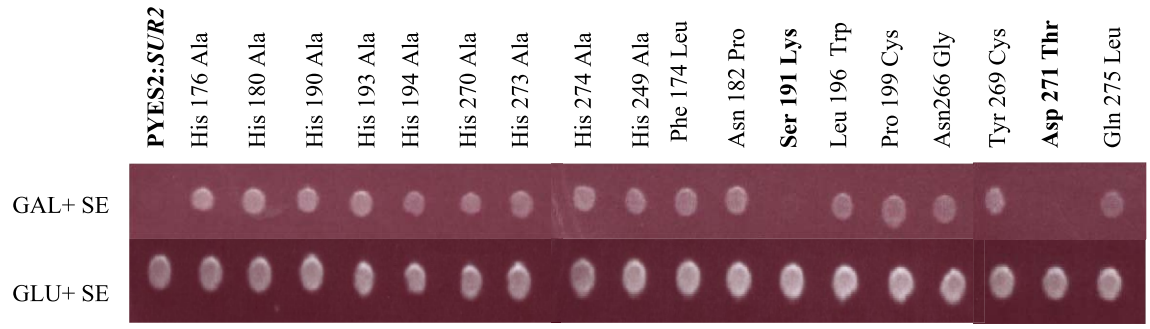

Fig. 5. Syringomycin E sensitivity of mutants. Cells were replica plated onto uracil dropout medium with glucose or galactose containing 1 μg/ml syringomycin E. Growth was examined after 24 h incubation at 28–30 °C.

corresponding normal hydroxylase activities of these mutants.

4. Discussion

In this work, we provide data that support Sur2p as a member of the family of diiron enzymes with active site histidine–rich motifs [29]. Hydropathy analysis for different proteins from this family indicates that these characteristic motifs are localized within the hydrophilic domains on the cytoplasmic face of the endoplasmic reticulum. Histidine-rich domains have been proposed to stabilize an active site metal center. Previous site-directed mutagenesis studies on these enzymes [17–19] focused on the desaturases. To our knowledge, the present report is the first structure–function study of the hydroxylases and the first such study of an enzyme for sphingolipid biosynthesis.

We were able to demonstrate that eight histidines are each crucial for C-4 sphingoid base hydroxylase activity. Conversion of each of the eight conserved histidines into an alanine residue failed to complement the yeast *sur2*Δ mutant. An additional conserved histidine residue (His 249) was shown to be essential for Sur2p catalytic activity. In studies by Taton et al. [17], the corresponding His residue from Erg3p was converted into a leucine or glutamic acid, and this significantly reduced the desaturase activity of this enzyme. It is possible that this additional histidine residue, located between the second and third histidine-rich cluster, is important for active site stability.

Another group of mutants was constructed by altering conserved residues in close proximity to the histidine cluster. These residues were converted into corresponding residues found in Erg3p. Hydroxylases and desaturases are proposed to be mechanistically very similar. Small changes in their active site geometries can shift the balance between hydroxylase and desaturase action. Broun et al. [28] showed that the active sites of a pair of diiron plant oleate desaturases and oleate hydroxylases are closely related. In addition to the eight histidines, there are seven highly conserved amino acids in oleate desaturases from several plant species. The functions of each of the seven residues were examined using a site-directed mutagenesis approach. Corresponding residues found in hydroxylases were replaced with residues from equivalent positions in oleate desaturases. Also residues found in oleate desaturases were replaced with corresponding hydroxylase residues. Four amino acid substitutions resulted in conversion of oleate 12-desaturase into a hydroxylase. A six-amino acid-substitution led to the conversion of the hydroxylase into a desaturase. In the present work, a similar active site modification study of Sur2p was undertaken. Mutants with single site replacements of hydroxylase residues with desaturase residues were shown to lose their hydroxylase activity but with no concomitant gain of desaturase activity. This indicates that the conserved residues are important for hydroxylase activity and their conversion into different amino acids failed to complement the Sur2p deletion mutant.

The conversions of Ser 191 into Lys and Asp 271 into Thr had no effect on the hydroxylase activity nor did they provide desaturase activity. This shows that these residues are not directly essential for the hydroxylase-specific catalysis and that the single substitutions are not sufficient for desaturase activity.

Studies by Broun et al. [28] showed that changes in catalytic activity resulted from a combined effect of several amino acids with partially overlapping effect. Following these studies, we constructed multiple site mutants using non-histidine residues. All the mutants had reduced levels of mutated Sur2p, and no mutant was able to synthesize desaturated sphingoid bases. Residues for multiple site mutants were chosen randomly, and it is possible that the combinations selected resulted in decreased protein stability.

It was shown by Ternes et al. [11] that the family of membrane-bound desaturases and hydroxylases with the tripartite histidine motif can be divided into two groups: either short or long spacing between the first and second histidine box. Sur2p has short spacing while proteins from the newly defined DES family of C4(5) sphingoid base desaturases have long spacing. It is possible that larger active site architectural changes are needed for interconversion of C-4 hydroxylase–desaturase activities, and this could explain the failure to convert Sur2p into a desaturase by alteration of only one or a few amino acids.

Additionally, we tested all mutants for their sensitivity to syringomycin E. The present results are in accordance with previous studies showing that C-4 hydroxylation is important for the antifungal action of syringomycin E. Mutants with defects in C-4 hydroxylated sphingoid base biosynthesis were resistant to this antifungal agent. It is known than syringomycin E targets plasma membranes and that sphingolipid and sterol biosyntheses are required for its action. Sphingolipids and sterols are major elements of lipid rafts [30–32], and sphingolipid hydroxylation is probably significant for proper assembly of rafts in the plasma membrane [33,34]. When yeast sphingolipids lack C-4 hydroxylated sphingoid bases, raft properties likely change in ways that influence syringomycin E action.

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