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ORIGINAL PAPER

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The sterol C-14 reductase encoded by the *Neurospora crassa erg-3* gene: essential charged and polar residues identified by site-specific mutagenesis

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Abstract Sterol C-14 reductase catalyses the reduction of the $\Delta^{14,15}$ bond in intermediates in the sterol biosynthesis pathway using NADPH as a cofactor. We have undertaken a systematic site-directed mutational analysis of all the conserved charged and potentially proton-donating residues of the sterol C-14 reductase from *Neurospora crassa*. The effect of each mutation was determined using an in vivo assay based on the complementation of the corresponding *N. crassa* mutant (*erg-3*). The non-complementing mutations were also tested in the *erg24* mutant of *Saccharomyces cervisiae*. The results are discussed with reference to the predicted topology of the enzyme and to its proposed catalytic mechanism, which involves addition of a proton from an appropriately positioned charged or polar residue to the substrate double bond, followed by addition of hydride ion from NADPH.

Keywords Lamin B receptor · Greenberg dysplasia · Smith-Lemli-Opitz Syndrome (SLOS) · Proton donor

Introduction

Sterol C-14 reductase is an integral membrane enzyme that catalyses the saturation of the $\Delta^{14,15}$ bond in sterol biosynthetic intermediates using NADPH as a co-factor. This reaction has been proposed to involve the electrophilic addition of a proton from an appropriately positioned charged or polar amino acid to the substrate double bond, to form a carbocationic intermediate (CCI). A hydride ion from NADPH is then added to the CCI to yield the reduced product (Taton et al. 1989). Morpholine, piperidine and azasterol fungicides inhibit this enzyme by mimicking the structure of the CCI (Mercer 1991). To identify amino acid residues that might be critically important for this reaction, we decided to systematically neutralize conserved charged and potentially proton-donating polar residues in the sterol C-14 reductase from *Neurospora crassa* and test the resulting mutants for the ability to complement the *erg-3* mutation (in the gene for the sterol C-14 reductase of *N. crassa*) Mutants that failed to complement in Neurospora were also tested for non-complementation of the corresponding *Saccharomyces cerevisiae* mutant (*erg24*). Similar approaches have been used by others to identify potentially important residues in two other sterol biosynthetic enzymes: sterol 8,7 isomerase (Moebius et al. 1999) and sterol C-5(6) desaturase (Taton et al. 2000).

Genes or cDNAs coding for sterol C-14 reductases have been sequenced from the filamentous fungi *N. crassa* (Papavinasasundaram and Kasbekar 1994), *Nectria haematococca* MPVI (D. P. Kasbekar and H. D. VanEtten, unpublished results), *Septoria lycopersici* (Aparna et al. 1998), and *Ascobolus immersus* (D. P. Kasbekar and G. Faugeron, unpublished results); from the yeasts *S. cerevisiae* (Lorenz and Parks 1992; Marcireau et al. 1992; Lai et al. 1994) and *Schizosaccharomyces pombe* (Smith 1995), and more recently from *Arabidopsis thaliana* (Schrick et al. 2000) and *Homo sapiens* (Holmer et al. 1998). The encoded proteins exhibit eight or nine putative transmembrane domains and share overall amino acid sequence identities in the range of 39% to 45%. The *Dictyostelium discoideum* cDNA project has reported a partial sequence of a cDNA clone, which suggests that it encodes a sterol C-14 reductase (http://www.csm.biol.tsukuba.ac.jp/CSM/SS/SSG792Q.Seq.d/). We determined the predicted primary sequence of this cellular slime mould protein for inclusion in a multiple sequence alignment.

Sterol C-14 reductases belong to an evolutionarily conserved family of proteins which includes the sterol C-7 reductases, sterol C-24(28) reductases and the

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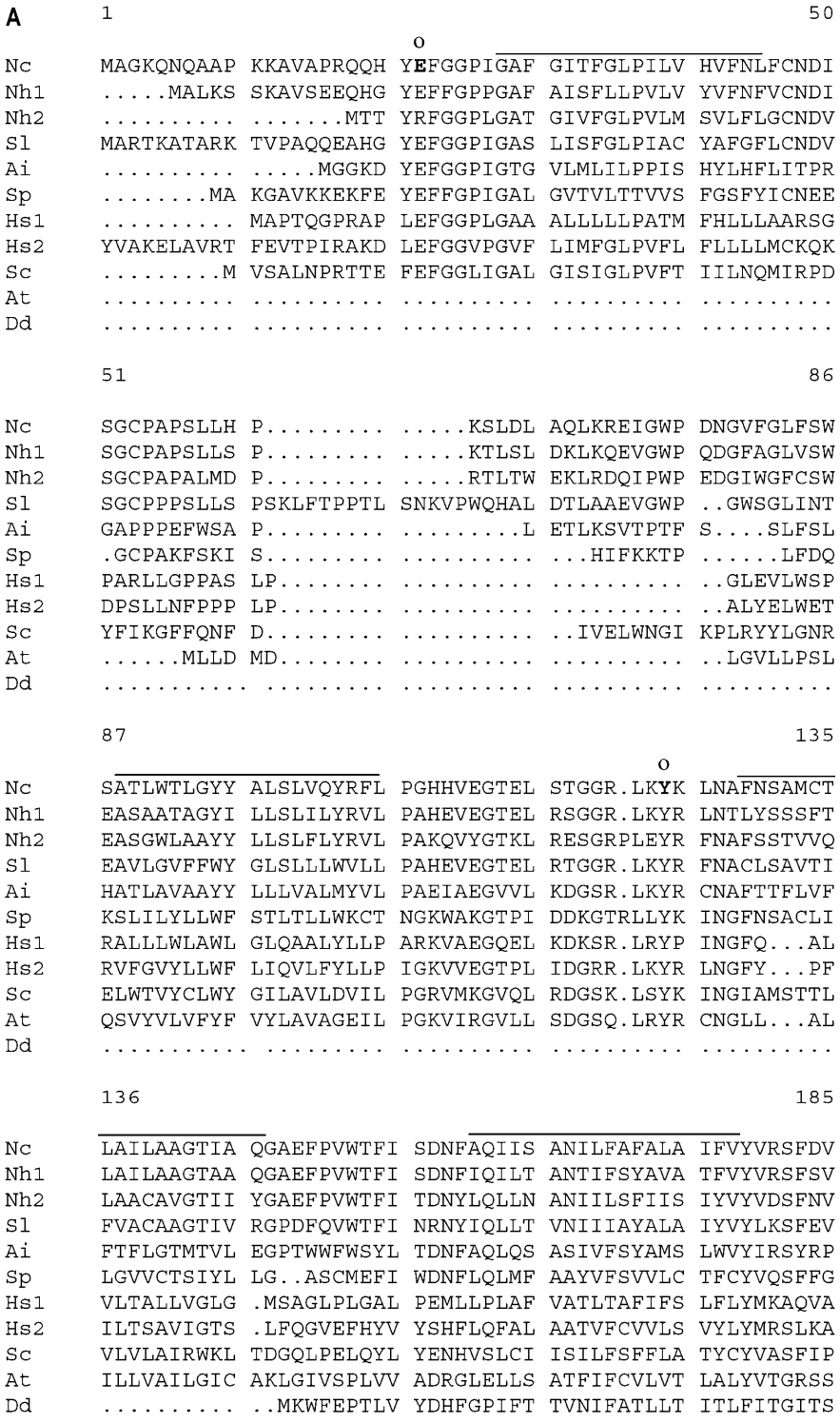

Fig. 1 Alignment of sterol C-14 reductase sequences from *Neurospora crassa* (Nc), *Nectria haematococca* MPVI (Nh1 and Nh2), *Septoria lycopersici* (Sl), *Ascobolus immersus* (Ai), *Schizosaccharomyces pombe* (Sp), *Saccharomyces cerevisiae* (Sc), *Homo sapiens* (Hs1 and Hs2), *Dictyostelium discoideum* (Dd) and *Arabidopsis thaliana* (At). Conserved and semi-conserved residues are indicated by *filled* and *open circles*, respectively. The residues modified in our study are indicated in *bold*. Potential membrane-spanning regions are *overlined*. The numbering refers to the Neurospora sequence

vertebrate lamin B receptors (LBR). LBR is a key player in the cell cycle-associated assembly and disassembly of the nuclear envelope in vertebrates. It is comprised of an N-terminal domain of approximately 200 residues that is highly basic and binds to lamin B, HP-1 type proteins and other nucleoplasmic factors, and a C-terminal domain that can function as a sterol C-14 reductase (reviewed by Kasbekar 1999). In a recent review, Waterham and Wanders (2000) have indicated that patients suffering from Greenberg skeletal dysplasia accumulate precursor sterols containing the $\Delta^{14,15}$ double bond, suggesting that they are defective in a sterol C-14 reductase activity. Mutations in sterol C-7 reductase are responsible for the autosomal recessive dysmorphogenetic disorder Smith-Lemli-Opitz Syndrome (SLOS) (Fitzky et al. 1998). Another human genetic disorder, desmosterolosis, is characterized by an accumulation of desmosterol and is probably due to a deficiency of sterol

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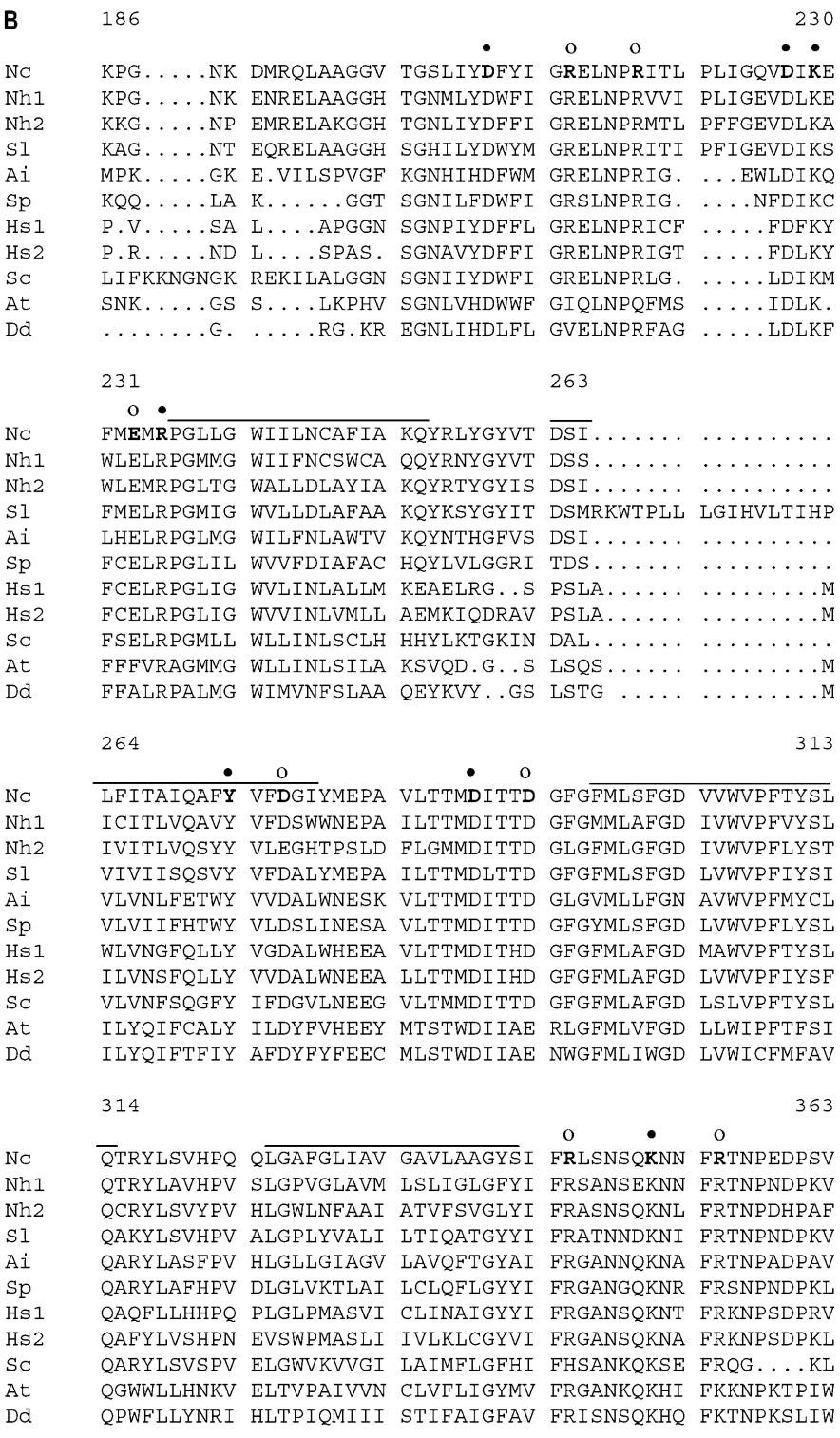

C-24(28) reductase activity (FitzPatrick et al. 1998). The identification of essential amino acid residues in the *N. crassa* sterol C-14 reductase might also help to pinpoint corresponding critical residues in other members of this protein family. To our knowledge, this is the first study aimed at identifying residues essential for sterol reductase activity.

Materials and methods

Strains and growth conditions

The wild-type *N. crassa* strains *74-OR23-1 mat A* (FGSC No. 987) and *OR8-1 mat a* (FGSC No. 988) and the mutant strain *erg-3 mat a* (FGSC No. 2725) were obtained from the Fungal Genetics Stock

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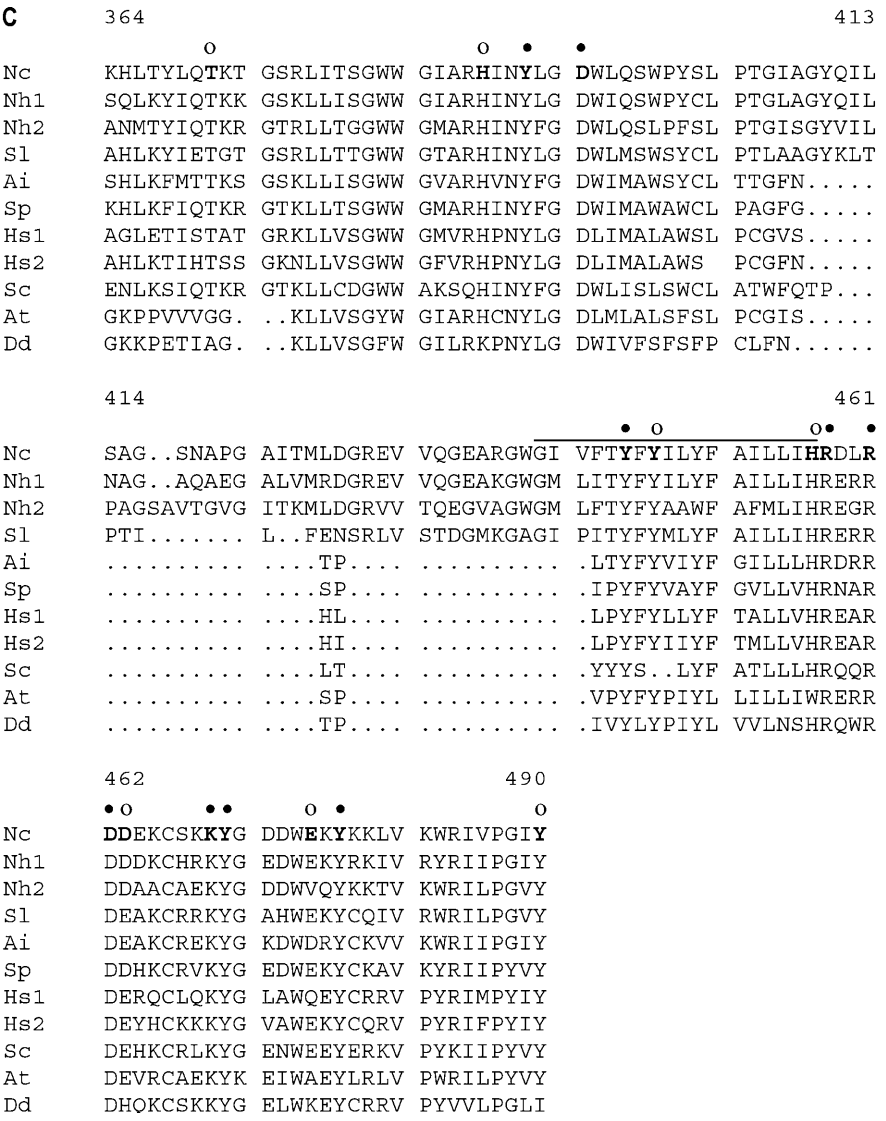

Center (FGSC, University of Kansas Medical Center, Kansas City, Kan.). The strains were maintained essentially as described by Davis and De Serres (1970). The *ERG24* null mutant strain of *S. cerevisiae*, AP2 (*erg24::LEU2 his3 leu2 trp1 ade2 lys2 ura3*), was obtained from Dr. Martin Bard (Indiana University-Purdue University, Indianapolis, Ind.). The *erg-3* mutant is sensitive to biochanin A and resistant to tomatine, whereas wild-type strains are resistant to biochanin A and sensitive to tomatine (Sengupta et al. 1995; Prakash et al. 1999). Complementation testing of the *erg-3* mutant phenotype was done by scoring for sensitivity to biochanin A and tomatine, as described by Prakash et al. (1999). Conidia were streaked onto 1.5% agar plates containing Vogel's medium N plus "sorbose" (0.05% fructose, 0.05% glucose and 2% sorbose) and supplemented with either α-tomatine (Sigma) at 90 μg/ml (from a 25 mg/ml stock solution in DMF) or biochanin A (Sigma) at 10 μg/ml. After an overnight incubation at 30°C, growth of only the *erg-3* mutant can be observed on tomatine-supplemented medium, and of only the wild type on biochanin A-supplemented medium. To obtain conidia, the strains were grown on 1.5% agar supplemented with Vogel's medium N and 1.5% glucose.

Hygromycin B (Sigma) was used for selecting transformants. It was added to the selection medium at 200 μg/ml (from a 100 mg/ml aqueous stock solution). Only strains expressing the *hph* gene could grow on hygromycin-containing medium.

Alignment of the available sterol C-14 reductase sequences, and site-directed mutagenesis of *N. crassa erg-3*

A multiple alignment of the sequences of sterol C-14 reductases from *N. crassa* (X77955), *N. haematococca* (X94315 and X98297), *S. lycopersici* (Y14389), *A. immersus* (Y10624), *D. discoideum* (AF308470), *S. cerevisiae* (M99419), *S. pombe* (L36039), *A. thaliana* (AF256536) and *H. sapiens* (L25931 and AF096304) was performed using the CLUSTALW program (available online at www.ebi.ac.uk/clustalw). This resulted in the identification of 16 strictly conserved and 16 semi-conserved charged and polar amino acid residues in the *N. crassa* sterol C-14 reductase (Fig. 1). The strictly conserved residues are D207, D227, K229, R235, Y273, D289, K351, Y391, D394, Y445, R458, R461, D462, K469, Y470 and Y477. The semi-conserved residues are E22, Y124, R212, R217, E233, D276, D293, R345, R355, T371, H388, Y447, H457, D463, E475 and Y490. Each of these residues was replaced either with a neutral residue of a similar size or with an alanine residue.

The plasmid pMOD86 contains a modified version of the *N. crassa erg-3* gene, designated mod 86, in which a *Pst*I site in the 3′ non-coding region of *erg-3* has been destroyed (Prakash et al. 1999). The modified gene can complement the *erg-3* mutant phenotype as effectively as the unmodified gene, and it was used for the

construction of the site-directed mutations. Single-stranded pMOD86 DNA was prepared by the protocol supplied with the Biorad in vitro mutagenesis kit (Catalog No. 170-3576). Briefly, CJ236 host cells were transformed with pMOD86. One colony was selected and grown in 2× yeast tryptone medium for 3 h. M13K07 helper phage was then added to the culture, and the incubation was continued for 10 h. Single-stranded DNA into which uracil had been incorporated was purified from the culture supernatant by PEG-6000 precipitation, phenol extraction and ethanol precipitation. Oligonucleotide-directed mutagenesis was performed following Kunkel (1985) using oligonucleotide primers synthesized by the in-house facility at CCMB. Table 1 lists the primers used to introduce the missense mutations. Single-stranded plasmid and mutagenic primer were denatured at 70°C for 5 min and allowed to anneal by slow cooling to 25°C. Extension of the annealed primer was done in a buffer containing 2 mM DTT, 6 mM $MgCl_2$ and 2 mM each of the dNTPs. T4 DNA polymerase and T4 DNA ligase was added to the reaction mix which was kept on ice for 5 min, and then incubated at 25°C for 5 min and finally at 37°C for 90 min. The mix was used to transform competent DH5α cells. Plasmid DNAs from four randomly picked colonies were sequenced using vector- and gene-specific primers, to confirm the presence of the introduced mutations and the absence of artifactual mutations. Automated sequencing was done using the Big Dye Terminator Ready Reaction kit (Perkin Elmer) and the ABI Prism 3700 DNA analyzer.

Transformation of *erg-3* conidia

The *erg-3* strain was grown for 7 days in Vogel's medium N supplemented with glucose, and the conidia were harvested in sterile water and filtered through cheesecloth. The conidia were pelleted in a Sorvall SS34 rotor at 4000 rpm for 10 min, washed twice in 30 ml of water and resuspended at a concentration of 3×10^9/ml. The plasmid DNA containing the mutagenised *erg-3* gene and the plasmid pCSN44 (Staben et al. 1989), which carries the selectable *hph* marker, was added to 40 µl of the conidial suspension and electroporated in a 0.2-cm cuvette (BTX). The electroporation conditions were 1.5 kV and 186 Ω. The time constant varied from 7 to 8.2 ms. After the pulse, 960 µl of 1 M sorbitol was added and 300 µl of the transformation mix was added to the top agar and plated on Vogel's-sorbose medium containing 200 µg/ml hygromycin. Transformants appeared after 3–5 days, and the frequency of co-transformation was generally above 90%.

UV absorption spectrum of sterols

Complementation was also tested by UV analysis of sterols in the transformants. Mycelia were grown in 30 ml of Vogel's-glucose medium for 3 days at 30°C, harvested by filtration, lyophilized, and disrupted with glass beads. About 50 mg of the mycelial powder was extracted for 7 to 8 h with 500 µl each of methanol and chloroform in a 1.5-ml microfuge tube. The organic supernatant was separated from the mycelial debris by centrifugation, washed once with 0.9% NaCl and twice more with 2 M KCl. The organic phase was dried in a rotary evaporator and redissolved in 10 µl of chloroform. Then 2 µl of this chloroform solution was diluted in 1 ml of ethanol and its UV spectrum (200 to 300 nm) was recorded using a Hitachi spectrophotometer. The dominant wild-type sterol, ergosterol, has UV absorption maxima at 272, 282 and 293 nm, whereas the sterols in the *erg-3* mutant absorb maximally around 250 nm (Prakash et al. 1999).

Testing for complementation of the yeast *erg24* mutant

The ORFs corresponding to each of the non-complementing mutant *erg-3* genes was cloned as a *Nco*I-*Spe*I fragment (*Nco*I blunt ended and *Spe*I with a 2-bp fill-in) from derivatives of pMOD86 into the *Sma*I/*Hin*dIII (with a 2-bp fill-in of the *Hin*dIII site) site of p426-TEF (Mumberg et al. 1995), a multicopy expression vector containing a *URA3* marker. The expression of the cloned fragment is driven by the strong *TEF* promoter. These plasmids were transformed into the AP1 strain (an *erg24* mutant) using the lithium acetate/ssDNA/PEG method (Gietz et al. 1992), and transformants were selected on synthetic complete medium lacking uracil. The transformants were checked for growth on YPD medium and their sterols were analysed by UV spectrophotometry. Sterols were isolated and analysed as described for *Neurospora*.

Table 1 Sequences of the mutagenic primers used in the study

Mutation	Sequence (5′→3′)[a]
E22Q	GGCCGCCAAACTGGTAGTGCTGC
Y124F	GTTCAGCTTGAACTTGAGCCGG
D207N	CAATGTAGAAATTGTAGATGAGG
R212A	ATTGAGCTCGGCACCAATGTA
R217A	CAGAGTGATGGCAGGATTGAG
D227N	TTCCTTGATGTTTACTTGGCC
K229A	CCATGAATTCCGCGATGTCTACT
E233Q	AGGGCGCATTTGCATGAATTC
E233D	AGGGCGCATGTCCATGAATTCC
R235A	AAGACCAGGGGCCATTTCCATG
R235K	AAGACCAGGCTTCATTTCCATG
Y273F	GTCAAAGACAAAGAAAGCCTGG
D276N	GTAGATTCCGTTAAAGACATAGAA
D289N	CGGTAGTGATATTCATCGTAGTC
D289E	CGGTAGTGATTTCCATCGTAGTC
D293N	CCCGAAGCCGTTGGTAGTGAT
R345A	GTTGGACAGGGCGAAGATGCTAT
K351A	CGAAAGTTGTTCGCCTGCGAGTTGGAC
K351R	CGAAAGTTGTTGCGCTGCGAGTTGGAC
R355A	GGGGTTCGTGGCAAAGTTGTTCTTC
T371A	TCCAGTCTTGGCCTGGAGGTAG
H388A	ATAATTGATGGCGCGAGCGATGCC
Y391F	GTCACCGAGAAAATTGATGTGG
D394N	CTGAAGCCAGTTACCGAGATAA
Y445F	GATGTAAAAGAAAAGTGAAAACGA
Y447F	GTACAAGATGAAAAAGTAAGTG
H457A	TGAGGTCGCGGGCGATCAAGAGA
R458A	CTCTGAGGTCGGCGTGGATCAAG
R458K	CTCTGAGGTCCTTGTGGATCAAG
R461A	TCTCGTCATCTGCGAGGTCGCGG
R461K	TCTCGTCATCCTTGAGGTCGCGG
D462N	ACTTCTCGTCATTTCTGAGGTC
D463N	GCACTTCTCGTTATCTCTGAGGTC
K469A	CGTCACCATACGCCTTGGAGCAC
Y470F	ATCGTCACCAAACTTCTTGGAGC
E475Q	CTTGTACTTCTGCCAATCGTCACC
Y477F	AAGCTTCTTGAACTTTTCCCAAT
Y490F	ATCGGGCTAGAAGATGCCAGGG

[a]The underlined nucleotides introduce the indicated mutation

Results and discussion

Site-directed mutagenesis and complementation of *erg-3*

Site-directed mutagenesis was used to replace each of the 32 conserved charged and polar amino acid residues in the *N. crassa* sterol C-14 reductase with either a neutral residue of a similar size or with an alanine residue (see Materials and methods). Plasmids containing the engineered *erg-3* genes were introduced, by co-transformation

together with the plasmid pCSN44, into conidia of the *erg-3* strain. The transformants were selected on hygromycin-supplemented medium and tested for complementation of the *erg-3* mutant phenotype. Twenty-one mutants (E22Q, Y124F, D207N, R212A, R217A, D227N, K229A, Y273F, R355A, T371A, Y391F, D394N, Y447F, H457A, D462N, D463N, K469A, Y470F, E475Q, Y477F and Y490F) were found to complement the *erg-3* mutation. For each of these engineered mutants we verified complementation in multiple hygromycin-selected transformants. Moreover, we never observed spontaneous reversion of the *erg-3* mutation. So we are quite confident that in each case the complementation is caused by the mutant allele. Eleven mutants (E233Q, R235A, D276N, D289N, D293N, R345A, K351A, H388A, Y445F, R458A and R461A) failed to complement the *erg-3* mutation. For the mutants that did not complement we confirmed the presence of the engineered allele in the transformants by PCR with primers specific for the transforming DNA (data not shown). Figure 2 presents the complementation data as determined by UV spectrophotometry for a representative set of mutants.

The complementing mutations thus identify conserved residues whose proton-donating ability does not appear to be involved in the enzymatic reaction. However, our assay does not rule out the possibility that these mutations might have subtle effects on enzyme activity that are below the limits of detection of this analysis. None of the complementing mutations altered the sensitivity of the transformants to the competitive inhibitor azasterol A25822B (data not shown).

For the mutants that failed to complement it was conceivable that conservation of charge at these positions might be sufficient for sterol C-14 reductase activity. For example, in some sterol C-14 reductases glutamic acid residues are present at the positions corresponding to residues D276 and D293 in the ERG-3 protein. Similarly, R345 and H388 are replaced by positively charged residues in some sterol C-14 reductases. To explore this possibility, we constructed six additional mutants (E233D, R235K, D289E, K351R, R458K and R461K) that introduced charge-conserving substitutions. Mutants E233D and R235K were able to complement *erg-3*, indicating that conserving the charge at these positions may be sufficient for enzyme activity. Four charge-conserving mutants (D289E, K351R, R458K and R461K) did not complement, which suggests that at these positions charge as well as the geometry of the group is important for sterol C-14 reductase activity.

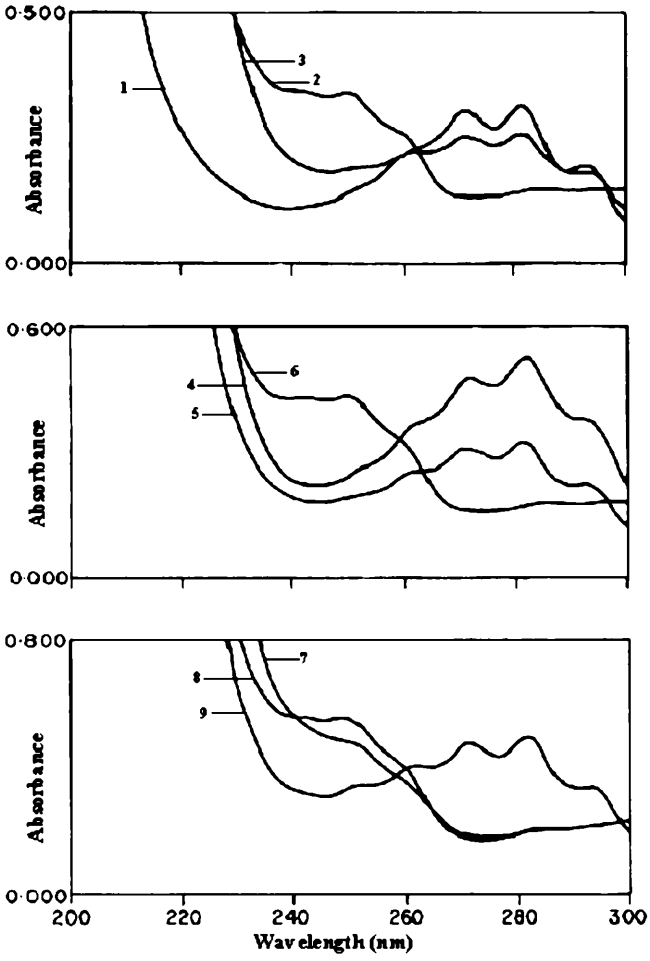

Fig. 2 Representative set of complementation data based on UV spectroscopic analysis of sterols from transformants of the *N. crassa erg-3* strain. *Top panel* Controls: (1) ergosterol; (2) untransformed *erg-3* strain; (3) mutant transformed with the wild-type *erg-3* allele. *Middle and lower panels* Mutants transformed with (4) K469A; (5) D394N; (6) K351A; (7)Y445F; (8) R461K and (9) E233D. Note that K469A, D394N, and E233D are able to complement *erg-3*, but K351A, Y445F, and R461K are not

Analysis of *erg-3* mutants for complementation of an *S. cerevisiae erg24* mutant

All the non-functional mutants, as well as the wild type *N. crassa* gene for sterol C-14 reductase and six complementing mutants (Y273F, T371A, Y447F, Y470F, Y477F and Y490F) were expressed in the yeast *erg24* mutant. The yeast *erg24* mutant is viable on synthetic medium but not on rich medium (Crowley et al. 1996). Yeast transformants expressing the wild-type sterol C-14 reductase of *N. crassa*, or any of the six complementing mutants, were able to grow on rich medium (Fig. 3), and to synthesize ergosterol, as judged by UV spectrophotometry (data not shown). In contrast, transformants with each of the non-complementing mutants, except K351R and R461K, were sensitive on rich medium and did not synthesize ergosterol. Yeast transformants bearing the K351R and R461K derivatives of *erg-3* grew very slowly on rich medium (Fig. 3). Analysis of the sterols in these transformants indicated the presence predominantly of the mutant sterol, with traces of ergosterol (data not shown). Thus the K351R and R461K mutations appear to result in extremely low

activity, which could be detected by over-expression in yeast but not in Neurospora.

Localization of critical residues in the predicted structure of sterol C-14 reductase

The program PHD (available at http://maple.bioc.columbia.edu/predictprotein) was used to determine the positions of the essential amino acids with respect to the overall topology of the protein. The predicted protein structure has nine transmembrane segments with the N-terminus present in the cytoplasm. The positions of the essential amino acids are indicated in Fig. 4. Of the eleven essential amino acid residues identified in this study, nine were in the presumptive cytoplasmic domains of the protein. This is in agreement with the observation that NADPH, which is required for activity, is present only in the cytosol. Hence the reaction probably takes place in the cytoplasmic leaflet of the membrane. A similar observation was made for the NADPH-dependent enzyme sterol 8,7 isomerase (Moebius et al. 1999). Some of the positively charged amino acids, like R235, R345, K351 and H388, present in the cytosolic loop may be involved in electrostatic interaction with the negatively charged 5′ phosphate and the 3′ phosphate groups of the co-factor.

Since the substrate for sterol C-14 reductase is hydrophobic, the reaction probably takes place within the membrane. Only two of the essential residues, D276 and Y445, lie within the membrane. However, D276 is replaced by glutamic acid in the *Nectria* sterol C-14 reductase, suggesting that it may not be a proton donor. Modifying the chain length of a proton donor should result in loss of activity. This suggests that Y445 might act as the proton donor. Interestingly, this residue is close to four highly conserved aromatic residues (F446, Y447, Y450 and F451). Hence, after addition of the proton to the C-14,15 double bond, the aromatic side chains of Y445 and/or of the neighbouring residues could stabilize the carbocation intermediate via a cation-π interaction. It has been established that interactions with the π-electrons of the aromatic rings represent an important mode of stabilization of cations in biological structures (reviewed in Ma and Dougherty 1997).

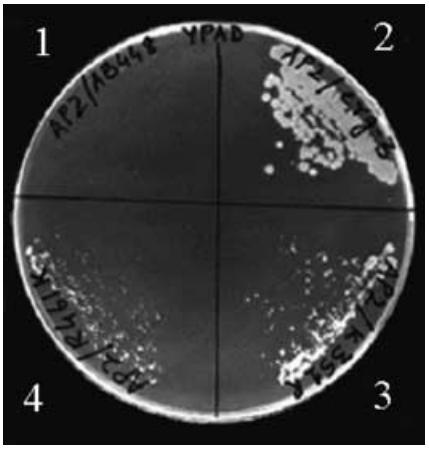

Fig. 3 Complementation of the *S. cerevisiae erg24* mutant strain AP2. The AP2 strain was transformed with (1) the empty vector pAB448, (2) wild-type *N. crassa erg-3*, (3) K351R, and (4) R461K and cells were streaked on rich YPAD medium. Note that much better growth is obtained for the transformant with the wild type allele than with the mutant derivatives K351R and R461K, and the cells transformed with the empty vector fail to grow

R235, R345 and R458 lie close to the predicted interface of the transmembrane segment and the loop. A positively charged residue located near the boundaries of transmembrane segments could act as a stop-transfer signal in the translocation of the transmembrane segment, and thus specify the topology of membrane proteins (reviewed in Dalbey 1990).

It has previously been suggested that a negative charge at residue 463 is essential for activity, because all the available sterol C-14 reductase sequences have either aspartic acid or glutamic acid at this position and the *fackel* mutation in Arabidopsis replaces the aspartic acid residue with lysine (Schrick et al. 2000). However, our results show that the D463N mutation can complement *erg-3*, thereby indicating that a negative charge is not essential at this position. Loss of activity in the

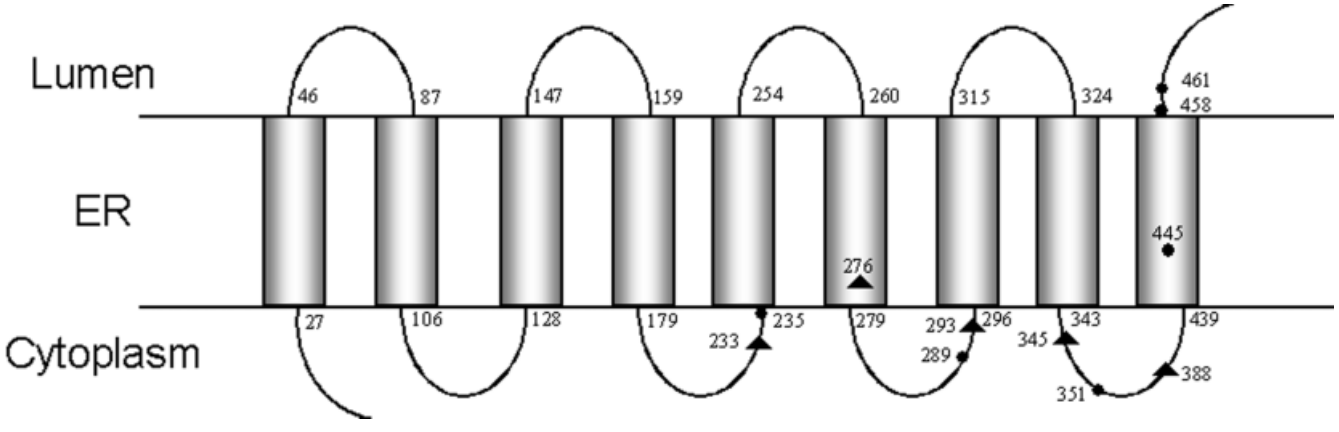

Fig. 4 Positions of the essential amino acid residues with respect to the predicted topology of the sterol C-14 reductase protein. The *filled circles* and *triangles* indicate the conserved and semi-conserved residues, respectively

Arabidopsis mutant may be due to the introduction of a drastically different residue rather than to charge neutralization.

Interestingly, except for E233, all the other essential amino acid residues identified in this study are also conserved among the other members of this protein family and they may be critical for their activity as well. In agreement with this possibility, residues corresponding to R235, R345, R458 and R461 in human sterol C-7 reductase are mutated in patients with Smith-Lemli-Opitz Syndrome (SLOS) (Waterham and Wanders 2000). Recently, it was shown that the rat sterol C-7 reductase has a sterol-sensing domain, which is also present in four other cholesterol-sensing proteins (Bae et al. 1999). The corresponding residues in the *N. crassa* sterol C-14 reductase are 159–355. If this stretch indeed functions as a sterol-sensing domain, then seven of the eleven essential residues fall within this domain.

We have identified charged and polar amino acid residues essential for sterol C-14 reductase activity by expression in the *erg-3* mutant of Neurospora, and corroborated the results by transformation in yeast. The absence of any structural information for this enzyme limits our ability to present any hypothesis about the specific roles played by the amino acids. The isolation of intragenic suppressors of the non-complementing mutants by selection on rich medium might help in identifying interacting residues. Biochemical analysis of the mutant sterol C-14 reductases will shed light on the roles these residues play in the structure and function of the enzyme. The significance of the sterol C-14 reductase activity of the lamin B receptor can also be addressed by substituting the residues identified as essential in this work.

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