Role of Highly Conserved Residues in the Reaction Catalyzed by Recombinant Δ^7-Sterol-C5(6)-Desaturase Studied by Site-Directed Mutagenesis

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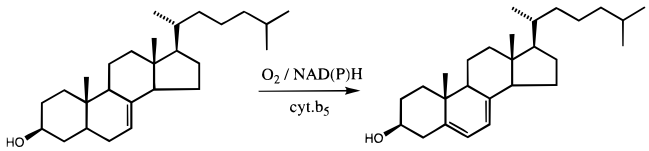
ABSTRACT: The role of 15 residues in the reaction catalyzed by *Arabidopsis thaliana* Δ^7-sterol-C5(6)-desaturase (5-DES) was investigated using site-directed mutagenesis and expression of the mutated enzymes in an *erg3* yeast strain defective in 5-DES. The mutated desaturases were assayed in vivo by sterol analysis and quantification of $\Delta^{5,7}$-sterols. In addition, the activities of the recombinant 5-DESs were examined directly in vitro in the corresponding yeast microsomal preparations. One group of mutants was affected in the eight evolutionarily conserved histidine residues from three histidine-rich motifs. Replacement of these residues by leucine or glutamic acid completely eliminated the desaturase activity both in vivo and in vitro, in contrast to mutations at seven other conserved residues. Thus, mutants H203L, H222L, H222E, P201A, G234A, and G234D had a 5-DES activity reduced to 2−20% of the wild-type enzyme, while mutants K115L, P175V, and P175A had a 5-DES activity and catalytical efficiency (*V*/*K*) that was similar to that of the wild-type. Therefore, these residues are not essential for the catalysis but contribute to the activity through conformational or other effects. One possible function for the histidine-rich motifs would be to provide the ligands for a presumed catalytic Fe center, as previously proposed for a number of integral membrane enzymes catalyzing desaturations and hydroxylations [Shanklin et al. (1994) *Biochemistry 33*, 12787−12794]. Another group of mutants was affected in residue 114 based on previous in vivo observations in *A. thaliana* indicating that mutant T114I was deficient in 5-DES activity. We show that the enzyme T114I has an 8-fold higher K_m and 10-fold reduced catalytic efficiency. Conversely, the functionally conservative substituted mutant enzyme T114S displays a 28-fold higher V_{max} value and an 8-fold higher K_m value than the wild-type enzyme. Consequently, *V*/*K* for T114S was 38-fold higher than that for T114I. The data suggest that Thr 114 is involved in stabilization of the enzyme−substrate complex with a marked discrimination between the ground-state and the transition state of a rate-controlling step in the catalysis by the 5-DES.

Δ^7-Sterol-C5(6)-desaturase (EC 1.3.3.2) (5-DES)[1] is membrane bound and catalyzes introduction of a C5 double bond into the B ring of Δ^7-sterols to yield the corresponding $\Delta^{5,7}$-sterols in mammals (*1*), yeast (*2*), and plants (*3*) (Figure 1). Enzymatic activity requires molecular oxygen, is strongly promoted by addition of NADH or NADPH, is inhibited by cyanide, hydrophobic metal chelators, and cytochrome *c*, and is insensitive to carbon monoxide. In addition, membrane-bound cytochrome b_5 is an obligatory electron carrier from NAD(P)H through NAD(P)H cyt b_5 reductase to the mammalian (*4*) and plant (*5*) 5-DES. The cDNAs or genes encoding 5-DES have been cloned in *Saccharomyces cerevisiae* (*6*), *Arabidopsis thaliana* (*7*), *Homo sapiens* (*8*, *9*), and *Candida glabrata* (Genbank L40390). These proteins differ slightly in size, and the *A. thaliana* 5-DES shares 29, 35, and 29% identity with the yeast, human and *Candida* 5-DES, respectively (Figure 2). Moreover, the desaturases possess a central portion containing three conserved histidine-rich motifs which are found in an important class of integral membrane enzymes that includes desaturases, hydroxylases, epoxidases, and acetylenases (*10*−*13*).



(1) (2)

FIGURE 1: Reaction catalyzed by Δ^7-sterol-C5(6)-desaturase.

Recently, we described an *A. thaliana* recessive mutant (*ste1*) accumulating Δ^7-sterols at the expense of Δ^5-sterols (*14*). This mutant was considered to be deficient in 5-DES. Alignments of the wild-type ORF with the *ste1* ORF revealed a single amino acid substitution: threonine 114 in the wild-type is changed to isoleucine in *ste1* (*9*). Expression of the corresponding ORFs in the yeast *erg3* null mutant revealed that the *ste 1* ORF was one-sixth as efficient as the wild-

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[1] Abbreviations: 5-DES, Δ^7-sterol-C5(6)-desaturase; cyt b_5, cytochrome b_5; GC-MS, coupled gas chromatography−mass spectroscopy; HPLC, high-pressure liquid chromatography; MS, mass spectroscopy; OD, optical density; ORF, open reading frame; PEG, poly(ethylene glycol); R_f, retention factor on thin-layer chromatography; TE, Tris EDTA; TLC, thin-layer chromatography; t_R, relative retention time; YNB, yeast nitrogen base; YPG, yeast peptone glucose.

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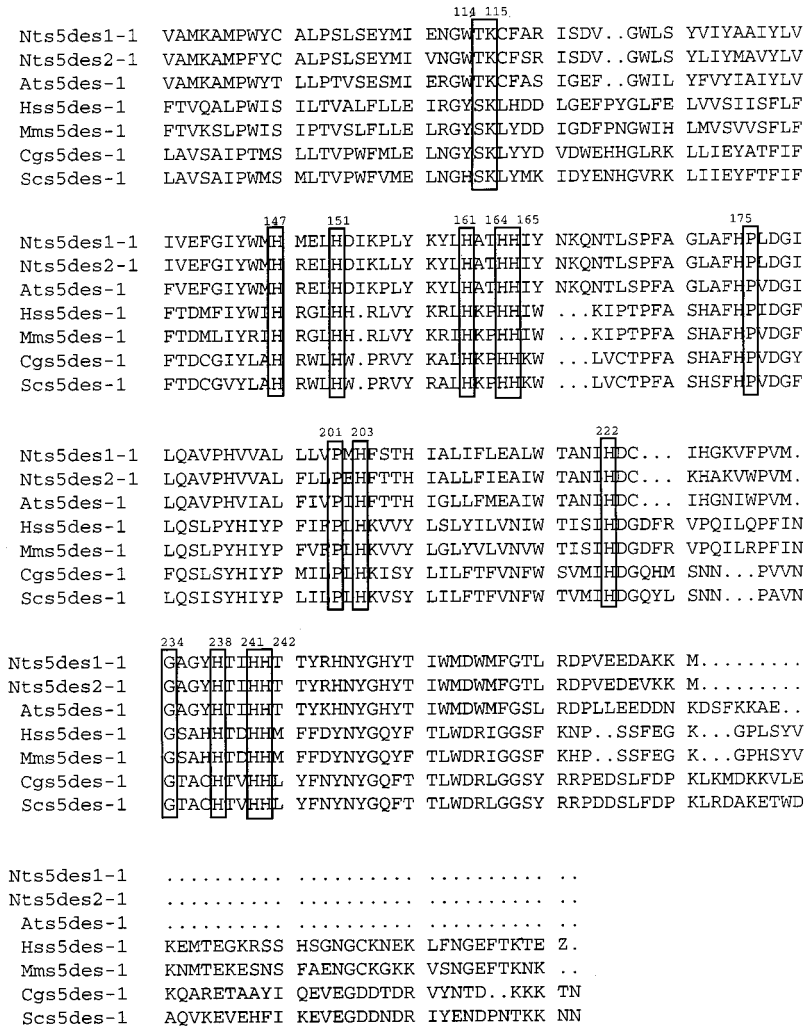

FIGURE 2: Sequence alignment of the Δ^7-sterol-C5(6)-desaturases: Alignment was performed using the PILEUP program of the GCG package run with default parameters. For more clarity partial sequences beginning at valine 90 of the Arabidopsis sequence have been shown. Nts5des **1** and **2** are *Nicotiana tabacum* sequences (Genbank accession AF081794 and AF099969), Ats5des is an *A. thaliana* sequence (Genbank accession X90454), Hss5des is the *H. sapiens* sequence (Genbank accession AF069469), Mms5des is the *M. musculus* sequence (Genbank accession AB016248), Cgs5des is the *C. glabrata* sequence (Genbank accession L40390), and Scs5des is the *S. cerevisae* protein (Genbank accession P32353). Mutated amino acids have been boxed. Numbers above the sequences refer to the corresponding mutated amino acid from the *Arabidopsis* sequence.

type ORF in the yeast biosynthetic pathway, supporting the view that the T114I mutation is responsible for the *ste 1* phenotype (*14*). So far, no information concerning residues essential for activity of this class of integral membrane sterol desaturases or an in vitro enzyme assay functional for the recombinant 5-DES in yeast microsomes and using the membrane bound cytochrome b_5 and cytochrome b_5 reductase has been described.

In the present study, we used site-directed mutagenesis to assess the importance for activity of each of the eight histidine residues of the three histidine-rich motifs, H147$X_{(3)}$-H151, H161$X_{(2)}$H164H165, H238$X_{(2)}$H241H242, and also of other evolutionarily conserved residues in the central part of the 5-DES such as K115, P175, P201, H203, H222, and G234. In addition, site-directed mutational analysis of residue 114 was conducted in order to probe the catalytic role of this functionally conserved residue. We then analyzed the effects of the mutations by assaying in vivo the mutant desaturases after expression in an *erg3* strain defective in 5-DES**,** by sterol analysis and quantification of $\Delta^{5,7}$-sterols. Second, we developed an in vitro enzyme assay allowing the effects of these mutations on microsomal 5-DES activity to be examined. To our knowledge, this is the first reported site-directed mutational analysis of a late enzyme in the plant sterol biosynthetic pathway and the first in vitro study of a mutated integral membrane lipid desaturase.

EXPERIMENTAL PROCEDURES

Materials. The following chemicals and reagents were purchased from Sigma: β-NADH (disodium salt), NAD^+, glutathione (reduced form), Trizma base, 5α-cholest-7-en-3β-ol (**1**), ergosta-5,7,9(11),22-tetraen-3β-ol **3**.

Strains. Genotypes of *Escherichia coli* DH5α and *S. cerevisiae erg3* (LB4-3A) have been described elsewhere (*7*).

Plasmids. A pBluescript SK^- vector (Stratagene) was used for subcloning the sterol-C5-desaturase cDNA and open reading frame (ORF). The pVT102U vector, kindly donated by Dr. T. Vernet (Genetic Engineering section, Biotechnology Research Institute of Montreal) was used in yeast transformation experiments. This plasmid contains an *E. coli*

replication origin, a yeast 2 μm replication origin, the *E. coli* ampicillin resistance gene, and the yeast gene *URA3* encoding an orotidine 5-phosphate decarboxylase for complementing uracil auxotrophy in recipient yeasts. The vector utilizes the constitutive alcohol dehydrogenase (ADH) promoter and terminator.

Arabidopsis Δ^7-Sterol-C5(6)-desaturase ORF Synthesis. Open reading frame synthesis from the *A. thaliana* ecotype *Columbia* Δ^7-sterol-C5(6)-desaturase obtained by PCR using a cDNA cloned from a silique library (*7*) was carried out using primer 324 (sense) 5′-gCCg **TCTAgA**ATggCggCggATAATgCTTATC-3′ beginning with an *Xba*I site and primer 325 (antisense) 5′-gCCg**CTCgAg**TCACTCTgCTTTCTTgAAgC-3′ beginning with an *Xho*I site. Amplification was performed using Pfu polymerase and corresponding buffer (Stratagene) in a final volume of 100 μL. The PCR was performed in a Mini cycler (Programmable Thermal Controller; Model PTC-150, MS Research, Inc.) starting with 5 min at 93 °C, then 30 cycles consisting of 1 min at 93 °C, 2 min at 55 °C, 3 min at 72 °C, and then a final step of 10 min of elongation at 72 °C. PCR products were separated on 1% (wt/vol) agarose gels.

Site-Directed Mutagenesis. The mutated alleles of the sterol-C5-desaturase were obtained by a technique based on the introduction of one or two point mutations in the DNA sequence as follows: two separate PCR reactions were performed with about 10−50 ng of the pBluescript vector containing the sterol-C5-desaturase ORF. The first reaction was realized with the primer 324 (sense) described above and a primer antisense ("AS") introducing the chosen mutation. The second PCR reaction was performed with a primer sense ("S"), complementary to the primer antisense introducing the mutation and the primer 325 (antisense) described above. After phenol/chloroform extraction and precipitation of the amplified fragments with 3 M NaOAc, pH 4.8, and purification from agarose (Prep-A-gene purification kit Biorad), the two fragments were hybridized due to the overlapping regions from the primers "S" and "AS" introducing the mutation. The hybridization was carried out in a final volume of 20 μL in the presence of PCR buffer (2 min at 100 °C, 20 min at 42 °C, and 20 min at room temperature). Finally, a PCR on 1 μL of the hybridization mix using primers 324 and 325 allowed the synthesis of the mutated open reading frame of the sterol-C5-desaturase.

Amplifications of DNA fragments were performed using Pfu polymerase and corresponding buffer (Stratagene) in a final volume of 100 μL in a mini cycler (described above). The amplification process began with 5 min at 93 °C and continued with 25 cycles consisting of the following: 1 min at 92 °C, 2 min at 56 °C, 3 min at 72 °C, and then 10 min elongation at 72 °C. The oligonucleotides used for mutagenesis are shown in Table 1.

Construction of an Expression Vector. The Δ^7-sterol-C5-(6)-desaturase mutant ORFs and wild-type *A. thaliana* ecotype Columbia ORF were used as 846 bp *Xba*I−*Xho*I fragments cloned into pVT102U for yeast transformation experiments.

Nucleotide Sequence Determination. The sequencing of PCR products was performed with an automatic sequencer (Perkin-Elmer model 373) using T3, T7, and SP6 primers and specific oligonucleotide sequences. A modified *Thermophyllus aquaticus* (Taq) polymerase capable of incorporating fluorescent dNTP's was used. Complete sequencing of both strands of DNA was performed.

Table 1: Synthetic Oligonucleotide Primers Used for Site-directed Mutagenesis[a]

mutation	oligonucleotide sequence (5′-3′) (sense)
H146L	ATTggATgC**T**CAgAgAgCTTCATgAC
H146E	ggTATTTATTggAT**gg**A**g**AgAgAgCTTCATgAC
H151L	gCACAgAgAg**CT**TCTTgACATTAAgCC
H151E	ggATgCACAg**g**AgAgCTT**g**A**A**gACATT AAgCCTCTC-
H161L	CTCTATAAgTATCTCC**TT**gCCACCCATCATA TC
H161E	gCCTCTCTATAAgTATCTC**g**A**A**gCCACC CATC
H164L	CTCCATgCCACCC**T**TCATATCTAC AACAAgC
H165L	CCATgCCACCCA**T**C**T**TATCTACAACAAgCAg
H238L	ATgggTgCAggATAC**CT**TACgATACACCAC ACg
H238E	gggTgCAggATAC**g**A**A**ACgATACACCACACg
H241L	ggATACCATACgATAC**T**CCACACgACATAC AAg
H242L	CCATACgATACACC**T**CACgACATACAAgC
H203L	gTgCCAATTC**T**TTTCACAACTCATATAgg
H222L	CggCgAACAT**CC**T**T**gACTgCATCCATggC
H222E	ggACggCgAACAT**C**g**A**AgACTgCATCCATggC
P175A	gAATACACTCTCT**g**CATTTgCCggg
P175V	gCAgAATACACTCTCT**gT**ATTTgCCggg CTTgC
P201A	gCTgTTTATAgTg**g**CAATTCATTTCAC
G234D	ggCCAgTAATgg**A**TgCAggATACC
G234A	ggCCAgTAATgg**CT**gCAggATACC
K115L	gAACgTggTTggACC**CT**ATgTTTTgCTAgC
T114S	gAACgTggTTgg**Ag**CAAATgTTTTgC

[a] Codons for the changed amino acids are underlined. Nucleotides represented in bold characters indicate the product point mutations.

Transformation of Yeast. Preparation of competent cells and transformation were performed according to ref *15* with some modifications.

Preparation of Competent Cells. A fresh culture with an initial OD of 0.2 was grown for 5 h in complete medium YPG [10 g/L yeast extract (Difco), 10 g/L bacto-peptone (Difco), 20 g/L glucose]. The cells were then successively pelleted by centrifugation (4000*g* for 5 min), washed twice in 1.5 mL of water, washed in 1.5 mL of a LiAc/TE solution (0.1 M LiAc, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.5), and finally resuspended in 200 μL of LiAc/TE solution.

Transformation. One microgram of plasmid DNA was mixed with 100 μg of salmon sperm DNA, sonicated 10 s, and heated to 100 °C for 20 min in a maximum final volume of 20 μL. Competent yeast cells (50−80 μL) and a solution of 40% PEG in LiAc/TE were added. The mixture was incubated 30 min at 30 °C, then 15 min at 42 °C. After centrifugation, cells were resuspended in 1 mL of YPG, incubated 1 h at 30 °C, collected, and then plated (with 100 μL of water) on minimal YNB medium [6.7 g/L yeast nitrogen base (Difco) and 10 g/L glucose] containing suitable supplements (histidine and adenine, 50 μg mL^{-1} of each).

Analytical Procedures. MS and GC-MS were determined at 70 eV with an MD800 Fison (U.K.) apparatus. The GC separation was carried out on a glass capillary column (WCOT: 30 m long x 0.25 mm i.d.) coated with DB1 (J & W Scientific, Folston CA). GC analyses were carried out with a GC instrument (model 8300, Varian, Les Ulis, France) equipped with a flame ionization detector at 300 °C, on a column injector at 250 °C and a fused capillary column (WCOT: 30 m long x 0.25 mm i.d.) coated with DB1 (H_2 flow rate of 2 mL/min). The temperature program used included a 30 °C/min increase from 60 to 240 °C and then a 2 °C/min increase from 240 to 280 °C. Relative retention times (t_R) are given with respect to cholesterol ($t_R = 1$).

HPLC analysis was carried out with a Waters S10 instrument equipped with a single pump and linked to an ultraviolet spectrophotometric detector at 281.5 nm. Separations were carried out with two analytical C18 ultrasphere 5 μm columns (250 × 4.6 mm) (Beckman) in line with a mobile phase of methanol:water (99.7:0.3 by vol) at a floumew rate of 1 mL/min at room temperature.

Sterol analysis. Total sterols of yeast (3−5 mg dry weight) were saponified in 500 μL of methanolic KOH (6% w/v) at 90 °C for 1 h. After addition of half a volume of water, total lipids were extracted three times with 1 vol of *n*-hexane. The dried residue was acetylated with a mixture of pyridine:acetic anhydride:toluene (1:1:1 v/v/v, 50 μL volumes) for 1 h at 70 °C. After evaporation of the reagents to dryness, the steryl acetates ($R_f = 0.5$) were purified by TLC on silica gel 60-F254 plates (Merck, Germany) using dichloromethane as developing solvent. Steryl acetates were analyzed by gas chromatography. Cholesterol was used as an internal standard for the quantification of their amounts. The steryl acetates were then identified by gas chromatography mass spectrometry (GC-MS) (*16*).

Microsomes Preparation. Erg3 cells transformed with a plasmid lacking an insert (pVT102U) or plasmids containing wild-type ORF (pVTORFwt) or mutant ORF's (pVTORFH146L and pVTORFH146E, etc.) were disrupted by glass bead homogenization (0.45 mm diameter) in 0.1 M phosphate buffer (pH 7.5) in the presence of reduced glutathione (1.5 mM) and nicotinamide (30 mM) four times for 3 min with 1 min interruptions at 4 °C. Cell debris, mitochondria, and nuclei were removed by centrifugation at 10000*g* for 20 min. Microsomes were isolated by centrifuging the supernatant at 100000*g* for 90 min and resuspending the pellet in an Elveljen-Potter homogenizer in 0.1 M phosphate buffer (pH 7.5) containing reduced glutathione (3 mM) and glycerol (20% vol/vol) to a final concentration of approximately 2.5 mg/mL protein.

Standard Assay for Recombinant Δ^7-Sterol-C5(6)-desaturases. Microsomes (0.40 ± 0.005 mL, 1 mg of protein) were incubated in the presence of 5α-cholest-7-en-3β-ol **1** (20−180 μM) emulsified in Tween 80 (final concentration 1.5 g/L), NADH (500 μM) and other additions as indicated in individual tables and figures.

Incubations were continued aerobically at 30 °C with gentle stirring for 90 min for all desaturases except mutant T114S, which was only incubated for 60 min. During this period the enzymatic rate was constant. Computer-assisted linear regression analysis gave correlation coefficients greater than 0.89 ($n = 6-7$). The reaction was stopped by the addition of 0.5 mL of 6% KOH in EtOH. A known amount (5.0 ± 0.1 μg for 0.4 mL incubation) of ergosta-5,7,9(11),-22-tetraen-3β-ol **3** was added as an internal standard, and the sterols were extracted three times with a total volume of 15 mL of *n*-hexane. The extracts were evaporated under argon at 25 °C. The resulting residue was suspended in 50 μL of absolute ethanol, and an aliquot (25 μL) was injected and analyzed by HPLC as described above. The cholesta-5,7-dien-3β-ol **2** produced (t_r relative to **3** in C18 HPLC = 1.42) was detected by recording the absorbance of the conjugated double bond at 281.5 nm and was readily separated from endogenous ergosterol **4** (t_r relative to **3** in C18 HPLC = 1.34) and cholesta-8,14-dien-3β-ol (t_r relative to **3** = 1.20), if present (Figure 3).

The amount of product formed was calculated by comparing the integrated peak areas of the known amount of added standard **3** and of the $\Delta^{5,7}$-product formed and corrected from the ϵ ratio at 281.5 nm between $\Delta^{5,7}$-sterol **2** and standard **3** [that is $\epsilon_{281,5}{}^{\Delta(5,7)}/\epsilon_{281,5}{}^{\Delta(5,7,9)} = 2.3$ for **2**, measured under the conditions of the assay]. The amount of product was eventually corrected for endogenous absorbance at the same t_R (if it was present) with the values obtained in the corresponding assay with boiled microsomes. Under these conditions the estimated limit of detection of the enzyme activity was 0.02 nmol h^{-1} mg^{-1}. The metabolite produced was collected and further identified by GC and GC-MS (*16*).

Enzyme Assays. Δ^8-Δ^7-Sterol isomerase assay was carried out in yeast microsomes as previously described (*17*). Sterol 14α-methyl-demethylase was assayed in the yeast microsomes as described in refs *18* and *19*. NADH-dependent cytochrome *c* reductase was measured as in ref *5*.

Miscellaneous. Membrane protein was determined as described by Bradford (*20*).

RESULTS AND DISCUSSION

Expression of the Wild-Type Arabidopsis thaliana Δ^7-Sterol-C5(6)-desaturase in the Yeast Null Mutant erg3: Assay for Desaturase Activity. The wild-type 5-DES ORF was transferred in the pVT102U vector and expressed in the *S. cerevisiae erg3* null mutant. The 5-DES activity was first qualitatively estimated in vivo by comparing the sterol profile of the *erg3* mutant and the transformants. When transformed with control plasmid pVT102U, *erg3* cells showed a sterol composition identical to that of the untransformed strain (data not shown). Transformants carrying the *A. thaliana* 5-DES wild-type ORF (pVTORFwt) contained up to 38% $\Delta^{5,7}$-sterols (ergosterol) in the total amounts of sterol (Table 2). Likewise, Δ^7-sterols decreased dramatically in these cells when compared to *erg3*. These data clearly indicated that the transformed cells exhibited high 5-DES activity in vivo.

To characterize the recombinant 5-DES activity in a more quantitative manner and to establish the kinetics of the desaturation reaction, we developed an enzyme assay for recombinant 5-DESs in yeast microsomes. Thus, 5α-cholest-7-en-3β-ol **1** (Figure 1) was incubated aerobically with microsomes of yeast null mutant *erg3* transformed with the wild-type 5-DES ORF or the control pVT102U vector, in the presence of NADH. The incubation allowed production of cholesta-5,7-dien-3β-ol **2** in the first case only. This product could be separated from the major endogenous sterol, ergosterol **4**, by HPLC analysis monitored at 281.5 nm (Figure 3). It was unequivocally identified as **2** by UV analysis, with coincidental retention times in GC and HPLC, and an electron impact spectrum identical to that of authentic standard (data not shown). The amount of **2** produced was quantified by comparison of the integrated HPLC peak areas of **2** and of a known amount of internal standard ergosta-5,7,9(11),22-tetraen-3β-ol **3** added prior to extraction and chromatography (Figure 3).

The present HPLC assay allowed the enzymological properties of recombinant *A. thaliana* wild-type 5-DES to be determined as shown in Table 3. Data indicate that the desaturation reaction has an absolute requirement for NADH and molecular oxygen. Coenzyme efficiency studies indicate

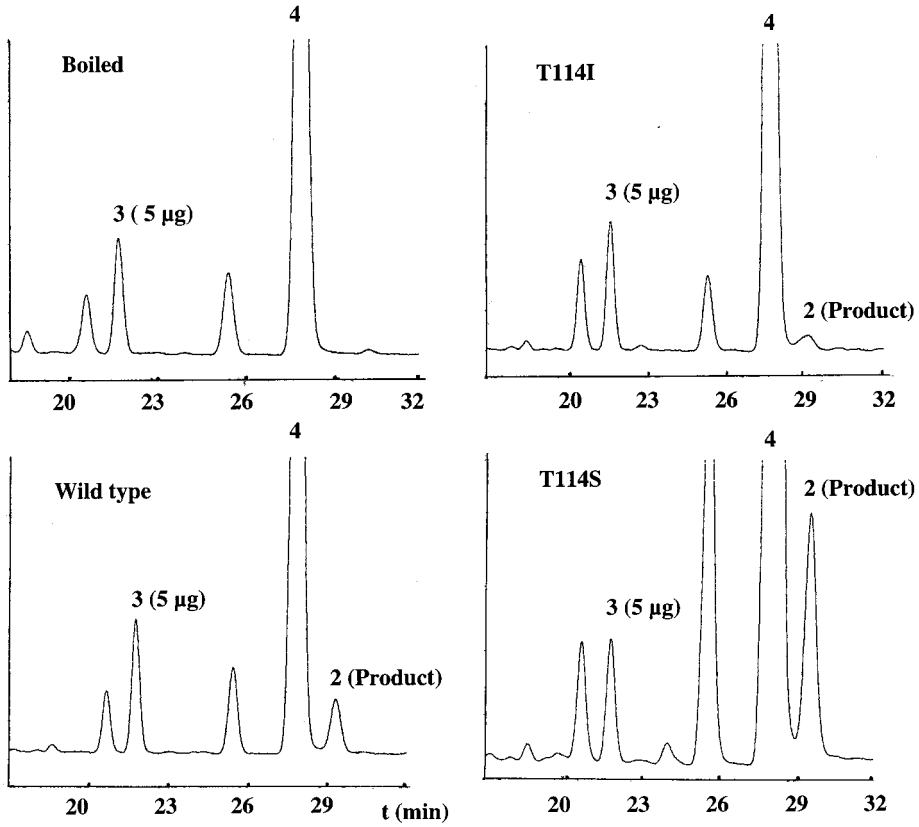

FIGURE 3: High-pressure liquid chromatography assay for wild-type and mutant T114I and T114S recombinant Δ^7-sterol-C5(6)-desaturases. After incubation of cholest-7-en-3β-ol **1** in the standard assay condition, the reaction mixture was extracted as described under Experimental Procedures and concentrated under Argon gas. An aliquot was injected into two analytical C18 ultrasphere HPLC columns joined in series and analyzed as described under Experimental Procedures; horizontal axis, time (mn); vertical axis, detector response (281.5 nm). The four profiles were obtained following identical incubation conditions with a 60 μM concentration of **1** and a similar concentration of microsomal proteins. Product is cholesta-5,7-dien-3β-ol **2**. Internal standard is ergosta-5,7,9(11),22-tetraen-3β-ol **3** (5.0 $\pm$ 0.1 μg). **4** is endogenous ergosterol. The peak at 25.6 min was identified as endogenous cholesta-8,14-dien-3β-ol. Substrate **1** is not detected at 281.5 nm. The boiled assay corresponds to the wild assay preparation.

that NADH is more efficient than NADPH. In addition, the desaturation is strongly inhibited by cyanide. The enzyme kinetics obtained with lipophilic substrate often deviate from the Michaelis−Menten-type reaction, and it has been suggested (*21*) that the classical Michaelis−Menten theory, which is applicable to water-soluble enzymes and substrates, may not be applicable to integral membrane enzymes and lipophilic substrates. To our knowledge, this experimental obstacle inherent to membrane-associated enzymes has not received a conclusive answer. In the present case, using our assay conditions (cf. Experimental Procedures), the obtained velocity/substrate concentration curves with respect to 5α-cholest-7-en-3β-ol **1** were almost linear. This showed that the usual Michaelis−Menten treatment was applicable to the recombinant 5-DESs and to **1** (Figure 4). However, the K_m and V_{max} values obtained (Table 3) are apparent and probably dependent on the membrane and detergent concentrations. Therefore, similar protein and constant detergent concentrations were used throughout the whole investigation, and we assumed that the assay conditions used would allow the kinetics of the active mutated 5-DESs to be compared. The measured values of K_m and V_{max} show that the recombinant *A. thaliana* 5-DES has a catalytic efficiency (V/K = 292 mL s^{-1} mg^{-1}) comparable to that measured for the native maize 5-DES (V/K = 250 mL s^{-1} mg^{-1}) (*3*) or yeast WA6 5-DES (V/K = 146 mL s^{-1} mg^{-1}) under similar experimental conditions (Table 4). These data suggest that the recombinant *A. thaliana* 5-DES is as good a substrate for the yeast ER-associated electron carrier system, i.e., cytochrome b_5 and NADH-cytochrome b_5 reductase (*4*, *5*, *22*), as for the plant electron carrier. Accordingly, cytochrome b_5 was detected by spectral measurement in the different *erg3* yeast microsomal preparations at a concentration range (100−200 pmol/mg of protein) comparable to that found in other yeast or plant microsomes (*3*, *23*). To our knowledge this constitutes the first in vitro functional recombinant plant membrane-bound lipid desaturation yeast system described so far. Moreover, the data show that the present heterologous expression system allows isolation of sufficient amounts of 5-DES for enzymological and mechanistic studies.

Choice of Residues for Mutagenesis. Multiple sequence alignment of Δ^7-sterol-C(5)6-desaturase across seven species [*A. thaliana*, two *Nicotiana tabacum* species (*9*) *C. glabrata*, *S. cerevisiae*, *H. sapiens* (*8*, *9*), and *Mus musculus* (Figure 2)] revealed that, across all seven species, proteins show identity in the three marked histidine-rich motifs, which are all situated in hydrophilic domains of the protein in the hydropathy profile. It is noteworthy that while the plant 5-DESs have three histidine-rich motifs, the yeast ERG3-ERG25 family shows a fourth histidine-rich motif. However, the role of this domain has not been clarified (*24*).

Similar histidine-rich motifs have been found in an extended family of integral membrane enzymes catalyzing desaturation, hydroxylation, epoxidation, and acetylenation

Table 2: Sterol Composition of Wild-Type Yeast, *Erg3* Null Mutant and Transformants Carrying the Mutagenized *A. thaliana* C5-Desaturases[a]

enzyme used to transform *erg3* null mutant	total $\Delta^{5,7}$-sterols[b] or complementation rate of *erg3* mutant	total Δ^{7}−sterols[c]	other sterols[d]
erg3 null mutant	0[e]	74.3[e]	25.7[e]
wild-type	38.0	18.8	43.2
H147L	0	81.8	18.2
H147E	0	76.7	23.3
H151L	0	62.9	37.2
H151E	0	76.3	23.7
H161L	0	81.1	18.9
H161E	0	76.0	24.0
H164L	0	87.1	12.9
H165L	0	90.5	9.5
H238L	0	77.3	22.7
H238E	0	76.8	23.2
H241L	0	83.4	16.6
H242L	0	74.9	25.1
H203L	5	66.9	28.1
H222L	<2.0	75.2	22.8
H222E	0	77.7	22.3
G234A	2.0	71.3	26.7
G234D	6.0	67.6	26.4
P201A	5.0	69.1	25.9
P175A	30.0	28.7	41.3
P175V	24.0	46.0	30.0
T114I	7.0	61.0	32.0
T114S	34.0	36.9	29.1
K115L	25.0	49.3	25.7

[a] Sterols were analyzed as their acetate derivatives as described in the Materials and Methods. [b] $\Delta^{5,7}$-sterols were exclusively ergosterol ($t_r = 1.24$). [c] Δ^{7}-sterols were a mixture of ergosta-7,24(24[1])-dien-3β-ol ($t_r = 1.31$), ergosta-7,22-dien-3β-ol ($t_r = 1.26$) and ergosta-7-en-3β-ol ($t_r = 1.32$). [d] Other sterols were lanosterol ($t_r = 1.35$), ergosta 8,24(24[1])-dien-3β-ol ($t_r = 1.28$), ergosta-8,22-dien-3β-ol ($t_r = 1.22$) and ergosta-8-en-3β-ol ($t_r = 1.29$). [e] Percentage of total sterols.

Table 3: Properties of Wild-Type *A. thaliana* Recombinant Δ^{7}-Sterol-C(5)6-desaturase

additions to microsomes		relative reaction rate (%)
	none	0
NADH	500 μM	100
NADH	500 μM + SR1[a]	96
NADH	1 mM + SR1	116
NADH	200 μM + SR1	105
NADH	50 μM + SR1	66
NADPH	500 μM	46
NADPH	1 mM + SR2[b]	50
NAD^+	500 μM	67
$NADP^+$	500 μM	14
CN^-		$I_{50} = 3\ \mu$M[c]
NADH	500 μM + argon + Glu + Glu 1-oxidase	0

[a] SR1: NADH regenerating system = EtOH (80 mM) + alcohol dehydrogenase (5 units). [b] SR2: NADPH regenerating system = Glu 6P (10 mM) + Glu 6P dehydrogenase (1 unit). [c] I_{50}: the inhibitor concentration which reduces the observed reaction rate by 50%. The data shown is the mean value obtained from two or more experiments.

(*10*−*13*). In one of these enzymes, rat stearoyl-CoA desaturase, eight His were shown to be essential for catalysis (*10*). In addition, spectroscopic studies of alkane ω-hydroxylase provided evidence that a diiron cluster is present in at least one member of this class of integral-membrane enzymes (*12*), and it was suggested that one possible role for these His residues may be to provide the ligands for the iron atoms.

To better understand the mode of action of the sterol-C5-(6)-desaturase, it was of primary interest to probe the role of the aforementioned eight His residues. Additionally, the seven desaturases show identity in a variety of amino acids in the domains between the histidine-rich motifs such as P175, P201, H203, H222, G234, and K115, that we also mutated. Finally, based on the view that the T114I mutation leads to the 5-DES deficiency in the *ste1* Arabidopsis mutant, we investigated the catalytic role of this functionally conserved residue.

Site-Directed Mutagenesis of the Δ^{7}-Sterol-C(5)-6-desaturase Gene. Mutation of Histidines 147, 151, 161, 164, 165, 238, 241, and 242. We first examined the replacement of the eight conserved histidine residues from the three motifs by sterically conservative leucine residues which are hydrophobic and electrically neutral. The effects of these mutations on the in vivo and in vitro activities of the *A. thaliana* 5-DES synthesized in the *erg3* null mutant cells are shown in Tables 2 and 3, respectively. The mutation of any of the eight conserved histidines by leucine totally eliminated the activity both in vivo, since no $\Delta^{5,7}$-sterols were detected (Table 2), and in vitro, since no 5-DES activity could be detected. Control experiments carried out in the same microsomal preparation indicated that both Δ^{8}-Δ^{7}-sterol isomerase (*17*), sterol 14α-demethylase (*18*, *19*) and NADH cytochrome *c* reductase (*5*) displayed similar activities as in yeast transformed with the wild-type 5-DES, attesting that the microsomes were functional (data not shown).

A number of soluble enzymes including stearoyl ACP desaturase (*11*, *25*), methane monooxygenase hydroxylase (*26*), and the R2 subunit of ribonucleotide reductase (RNR-R2) (*27*) contain a nonheme diiron center. In this class of enzymes, the terminal ligands for each iron atom comprise one histidine and one monodentate carboxylate in addition to bridging carboxyl groups. Recently, a site-directed mutant of RNR-R2, in which a single aspartate iron ligand (D84) was modified to a functionally conservative glutamate, has been described (*28*). This substitution resulted in a pronounced change in reactivity due to a greater stability of a peroxodiiron-(III) intermediate. Since replacement of histidines by arginines led to inactive Δ^{12}-desaturase in *Synechocystis* (*11*), we further probed the role of the above-mentioned histidine-rich motifs by mutating four of these histidines to glutamic acid residues which could provide ligands for a possible Fe center and change the reactivity of the 5-DES. However, these substitutions led to completely inactive 5-DES mutants, which failed to give detectable amounts of $\Delta^{5,7}$-sterols and had no 5-DES activity in vitro.

-Recently, by site-directed mutagenesis, eight conserved His residues in the rat stearoyl-CoA desaturase were individually converted into Ala. Each of these mutations failed to sustain the growth of the yeast *ole 1* mutant that was defective in Δ^{9}-desaturase, indicating that the conserved His residues were essential for catalytic function. It was suggested that they would in particular act as ligands for the iron atoms contained in the enzyme (*10*). Similarly, four His residues that are conserved in the *desA* gene for the Δ^{12} acyl-lipid desaturase from the cyanobacteria *Synechocystis* and are located within the three conserved histidine-rich motifs were replaced by arginines, and the mutated *desA* genes were overexpressed in *E. coli*. All of these mutations eliminated the Δ^{12}-desaturase activity, indicating that they are also

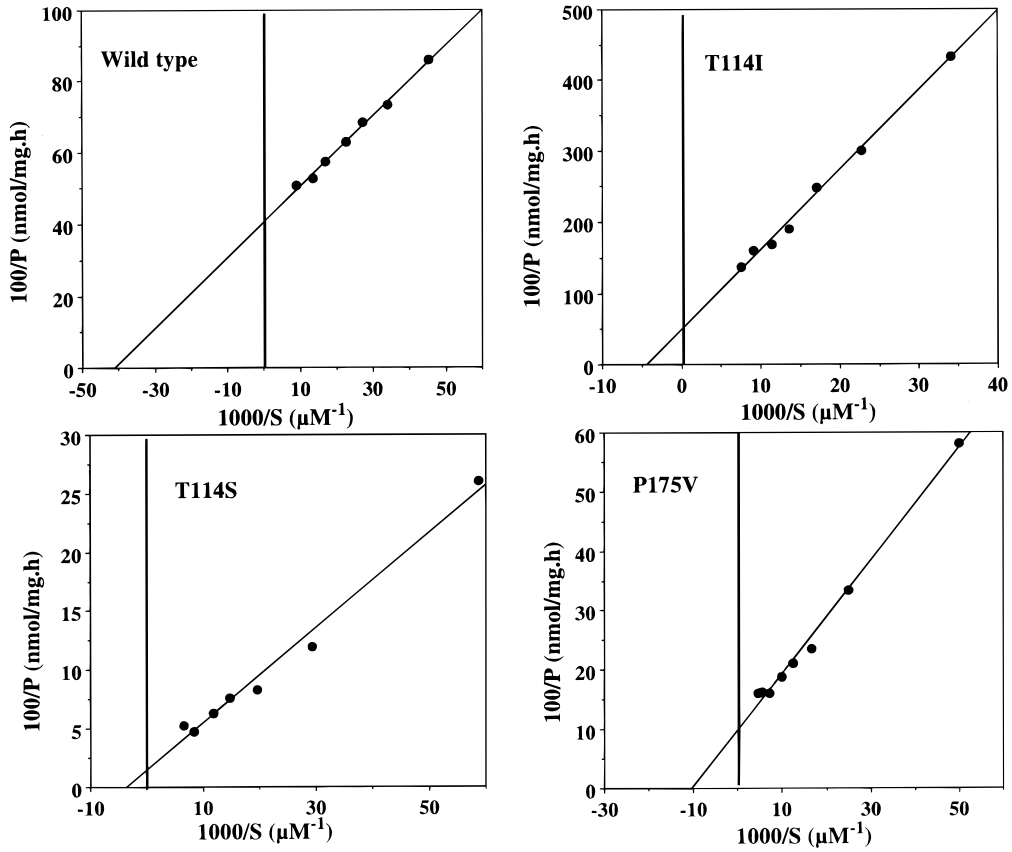

FIGURE 4: Double reciprocal plot of 5α-cholest-7-en-3*β*-ol (**1**) C5(6)-desaturation by microsomes of *S. cerevisiae* expressing wild-type *A. thaliana* Δ^7-sterol-C5(6)-desaturase and mutants T114I, T114S, and P175V enzymes.

essential for Δ^{12}-desaturation, perhaps by providing the ligands for the catalytic Fe center (*11*). A possible role for the three histidine-rich motifs of the 5-DES, consistent with the results of the present mutagenesis study and previous biochemical data indicating inhibition of the 5-DES activity by hydrophobic metal chelators (*3*), could also be to provide ligands for a presumed catalytic Fe center.

It could be that mutations may reduce 5-DES expression by changing synthesis or rendering the protein unstable due to misfolding or affecting its insertion into the membrane. In contrast to the rat liver stearoyl Δ^9-desaturase, the plant membrane-bound 5-DES has not yet been purified. Purification of the latter would have allowed production of antibodies to verify whether the expression levels of the mutated 5-DESs are similar to that of the wild-type enzyme. Along these lines, we wish to point out two recent studies performed in heterologous yeast systems closely related to ours. First, a series of site-directed mutations of membrane-bound human emopamil binding proteins (hEBP), which exhibit sterol Δ^8-Δ^7-isomerase activity upon heterologous expression in a sterol Δ^8-Δ^7-isomerization-deficient *erg2-3* yeast strain, have been described (*29*). Second, eight conserved histidines in the rat liver Δ^9-desaturase were mutated, and the ability of the mutated desaturase to complement the yeast *ole 1* mutant strain deficient in the Δ^9 -desaturase reaction was examined (*10*). Although, in both cases, the analysis of residues was performed only in vivo, no major differences in the expression level of mutants, which would cause a major loss of catalytic activity, were observed. Interestingly, none of the 64 described mutants of the abovementioned hEBP had not detectable isomerase activity (*29*). It has been shown in the case of another membrane-bound protein linked to sterol biosynthesis, i.e., obtusifoliol 14-demethylase (CYP51), that the length and structure of the hydrophobic segments anchoring the protein in the membrane were important for optimal expression, membrane location and accumulation, and functional expression of such integral-membrane proteins (*18*). The aforementioned eight histidines are all situated in hydrophilic domains of the protein in the hydropathy profile. The membrane-spanning segments of the corresponding mutants are thus identical to that of the wild-type 5-DES and it reasonable to think that the histidine replacements should not influence membrane location or accumulation of these mutants. Considering these data together, it seems unlikely that the lack of activity in *all* the 5-DES mutated in one of the eight histidines from the three motifs would result from a lack of expression and location in the yeast membranes. To get more information on this last issue, we examined whether mutation of other conserved residues would abolish 5-DES activity by studying the replacement of six of them, including two histidines not situated in the histidine-rich motifs.

Mutation of His 203, His 222, Pro 201, Pro 175, Gly 234, and Lys 115. Replacement of His 203 by a leucine residue caused a 7-fold decrease in 5-DES activity (Table 4) and led to a 5% percentage of $\Delta^{5,7}$-sterols (Table 2) in contrast to the 38% of $\Delta^{5,7}$-sterols found in the *erg3* deficient yeast strain transformed with the wild-type enzyme. Replacement of His 222 by a leucine had more pronounced effects on desaturase activity: the enzymatic activity was below the limit of detection, and only traces of $\Delta^{5,7}$-sterols could be detected in vivo. Moreover, the substitution of His 222 with

Table 4: Kinetic Parameters of *A. thaliana* C5-Desaturase Mutants and Wild-Type Enzyme Expressed in *S. cerevisiae* for Δ^7-Cholestenol

enzyme	$K_{m(app)}$ (μM)	$V_{max(app)}$ (nmol/mg x h)	$(V_{max})_{mut}/(V_{max)\ WT}$	V_{max}/K_m (mL/s x mg)	$\Delta(\Delta G_S)^a$ (kJ/mol)	$\Delta(\Delta G_T^{\#})^b$ (kJ/mol)	$\Delta(\Delta G^{\#})^c$ (kJ/mol)
wild-type	32 ± 10^d	2.6 ± 0.2^d	1	292	0	0	0
H 147 L		<0.02	<0.007				
H 147 E		<0.02	<0.007				
H 151 L		<0.02	<0.007				
H 151 E		<0.02	<0.007				
H 161 L		<0.02	<0.007				
H 161 E		<0.02	<0.007				
H 164 L		<0.02	<0.007				
H 165 L		<0.02	<0.007				
H 238 L		<0.02	<0.007				
H 238 E		<0.02	<0.007				
H 241 L		<0.02	<0.007				
H 242 L		<0.02	<0.007				
H 203 L	nd	0.35	0.13				
H 222 L		<0.02	<0.007				
H 222 E		0.17	0.06				
G 234 A		<0.02	<0.007				
G 234 D	nd	0.06	0.02				
P 201 A	nd	0.54	0.20				
P 175 A	70 ± 10	2.7 ± 0.5	1.0	139	+2.0	+1.9	−0.1
P 175 V	86 ± 11	11.2 ± 1.0	4.3	474	+2.5	−1.2	−3.7
T 114 I	240 ± 13	1.9 ± 0.1	0.73	28.5	+5.1	+5.9	+0.8
T 114 S	244 ± 28	73 ± 5	28	1077	+5.1	−3.3	−8.4
WA6; (S114)	37 ± 6	1.5 ± 0.2		146			
K 115 L	90 ± 10	6.2 ± 1.0	2.4	248	+2.6	−0.4	−2.2

[a] $\Delta(\Delta G_S)$: Apparent difference in the free energy of the enzyme−substrate complex between wild-type and mutant enzyme, calculated from $(\Delta(\Delta G_s) = RT \ln[(K_m)_{mut}/(K_m)_{WT}]$ (*45*). [b] $\Delta(\Delta G_T^{\#})$: Apparent free-energy changes for the mutant enzyme relative to the wild-type enzyme for the transition state of the desaturation reaction calculated from $\Delta(\Delta G_T^{\#}) = -RT \ln[V/K)_{mut}/(V/K)_{WT}]$ (*46*). [c] $\Delta(\Delta G^{\#})$ Apparent free energy changes for the desaturation of Δ^7-cholesterol for the mutant enzymes relative to the wild-type enzyme. $\Delta(\Delta G^{\#}) = \Delta G_T^{\#} - \Delta G_S = -RT \ln[V_{max,mut}/V_{max,WT}]$ (activation energy of the chemical step of bond making and braking). [d] The kinetic parameters are the average of two to four separate determinations. [e] nd: not determined.

glutamic acid led to a reduction of enzyme activity to 7% of that of the wild-type enzyme in vitro, and no $\Delta^{5,7}$-sterols were detected in vivo.

Substitution of proline residues may cause refolding of loops and change accessibility to the active site or modify the stability of the desaturase. In this respect, it was of interest to replace conserved proline residues by another nonpolar hydrophobic residue: either a smaller one such as alanine or a similar one such as valine. Mutation P201A reduced activity to 20% of that of the wild-type enzyme, and mutation P175A decreased substrate affinity without affecting maximum velocity of the desaturase. Additionally, replacement of Pro 175 by a valine residue increased about 4-fold the V_{max} and 3-fold the K_m of the mutated desaturase thus only slightly affecting the catalytic efficiency expressed as V_{max}/K_m, as shown in Table 4 and displayed graphically in Figure 5 for the most active mutant enzymes. The in vitro data were confirmed by the observed in vivo $\Delta^{5,7}$-sterol content of P201A, P175A, and P175V since P201A led to a low $\Delta^{5,7}$-sterol content (5%), while P175A and P175V displayed high $\Delta^{5,7}$-sterol contents. The higher catalytic efficiency and maximal velocity of P175V when compared to P175A was also reflected by a 3.8-fold higher amount of ergosterol detected in the membrane preparation of P175V than that found in P175A (data not shown). The different effects of substitution of Pro175 and Pro201 could reflect their distinct environments: while Pro 201 is situated in the middle of hydrophobic domain of the 5-DES protein in the hydropathy profile, Pro 175 is at the border between a hydrophobic and a hydrophilic domain. Substitution at the latter position could lead to more global conformational changes of the protein and of its active site, affecting indirectly the activity recovered in the microsomal fraction.

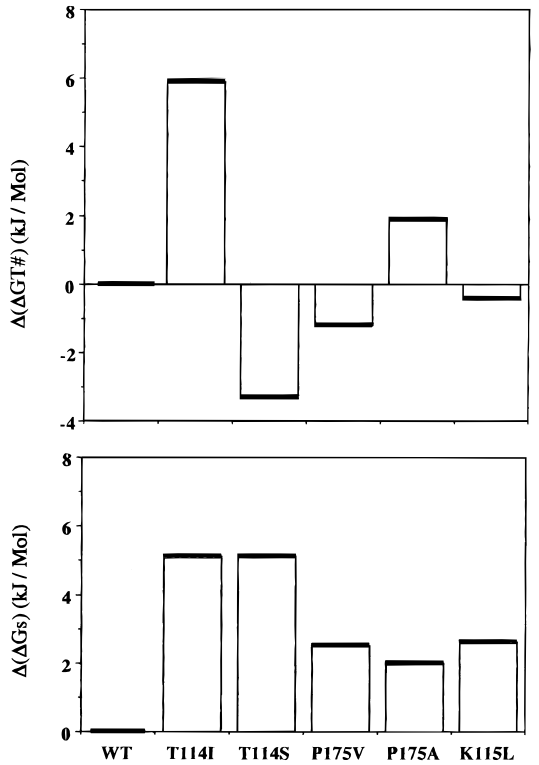

FIGURE 5: Comparative apparent free-energy changes for the recombinant cholest-7-en-3β-ol C5(6)-desaturase mutants relative to the wild-type enzyme for the enzyme substrate complex in the ground-state $\Delta(\Delta G_s)$ and in the transition state $\Delta(\Delta G_T^{\#})$ calculated as in Table 4.

In addition, Gly 234 was replaced either by a bigger nonpolar hydrophobic residue alanine or by a functionally distinct and negatively charged aspartic acid residue. Mutations G234A and G234D substantially reduced the 5-DES activity, and the corresponding $\Delta^{5,7}$-sterols contents were low.

Due to the simple observation that it is conserved in all 5-DESs known so far, it was expected that the replacement of Lys 115 should lead to a variation in activity. Replacement of Lys 115 by a hydrophobic neutral leucine residue increased the V_{max} for K115L 2.4-fold, indicating that this residue is not essential for enzymatic activity and that a positive charge is not required at position 115. K_m also increased 2.8-fold, and consequently, *V*/*K* for K115L was 80% of that for the wild-type enzyme. Thus, weakening of the transition-state binding was largely matched by increasing K_m, allowing a significant increase in the rate. Accordingly, the in vivo $\Delta^{5,7}$-sterols content of mutant K115L was one of the highest. Thus, data obtained with mutant K115L indicates that a positive charge is not required at position 115 and that this replacement apparently did not affect the recovery of the 5-DES activity in the yeast microsomal fraction.

Taken together, the results indicate first a good correlation between the in vivo and in vitro data. Second, although replacement of His 209, His 222, and Gly 234 lowered 5-DES activity, none of the six mutations in contrast to mutations of the eight histidines from the three motifs eliminated the 5-DES activity. Moreover, mutations at P175 and K115 gave 5-DES featuring catalytical efficiencies similar to or higher than that of the wild-type 5-DES.

Mutation of Threonine 114. As recently reported (*9*), the ORF from the *A. thaliana ste1* mutant contains a single amino acid substitution, T114I, which is most probably responsible for the 5-DES deficiency of this mutant. Upon the basis of these results, we investigated the role in catalysis of this important residue which is situated far outside the histidine regions. Thr 114 is not conserved in the *ERG3* family but the functionality is conserved since 5-DES from *C. glabrata*, *S. cerevisiae*, *M. musculus*, and *H. sapiens* have a serine in this position (Figure 2). Thus, it was of interest to replace Thr 114 by the functionally conservative serine residue and to study the catalytical properties of mutant T114I and T114S in comparison to the wild-type 5-DES. The kinetic parameters of mutant T114I and T114S are shown in Table 4 and Figure 5. Both mutants have a K_m increased approximately 8-fold in comparison to the wild-type 5-DES. In contrast, substitution at T114 had pronounced distinct effects on V_{max}. For T114I, V_{max} was slightly decreased, while for T114S, V_{max} increased 28-fold in comparison to the wild-type enzyme. Consequently, *V*/*K* for T114S was 38-fold higher than that for T114I.

The data were confirmed by the observed in vivo $\Delta^{5,7}$-sterol contents, since T114S led to the highest amount of $\Delta^{5,7}$-sterols (34%), while T114I led to only 7% of $\Delta^{5,7}$-sterols. The results indicate that the T114 residue strongly affects the reactivity of the desaturase but is not essential for catalysis. The fact that the K_m of the desaturase is increased in both mutants indicates that Thr 114 contributes to the stabilization of the enzyme−substrate complex in its ground state. Second, Thr 114 has an important role in stabilizing the transition state of a rate-controlling step of the 5-DES reaction, which is destabilized by 5.9 kJ/mol for T114I and stabilized by 3.3 kJ/mol for T114S compared with the wild-type desaturase (Table 4) (Figure 5). Replacement of Thr 114 by a functionally nonconservative isoleucine led to an approximately uniform increase in the free energy of both states in which the enzyme is bound to the Δ^7-sterol, i.e., the substrate−desaturase complex and the transition-state−desaturase complex. Thus, the rate of the reaction was but slightly affected. In this respect, T114I would represent a uniform binding mutant according to a previously proposed classification of mutation (*30*). In contrast, it is readily apparent that in the case of mutation of Thr 114 by a serine residue there is a 5.1 kJ/mol decrease in the apparent binding energy of the substrate in the ground state (ΔG_s) and 3.3 kJ/mol of additional stabilization of the transition state ($\Delta G_T^{\#}$), thus, leading to a 8.4 kJ/mol decrease in the activation energy ($\Delta G^{\#}$) required to reach the transition state and a substantial improvement of the desaturase reaction rate. Therefore, mutant desaturase T114S would represent a mutant modified both in binding and in catalysis of the elementary step (*30*). In this respect, it would be interesting to find out whether mutations at T114 preferentially alter the transition state of the rate-limiting chemical step in the whole desaturase reaction sequence. In addition, it would be interesting to investigate whether mutation of a residue involved directly in the catalytical process of the 5-DES would produce a new product. However, no significant new products for structural characterization were formed by the T114 mutants in this study. HPLC analysis of the assays performed with mutants T114S or T114I, when compared to the control assays devoid of 5-DES activity, revealed no new peaks, aside from the product peak **2**, at either 281.5 or 210 nm.

Incidentally, mutation T114I in mutant *ste 1* results from the in vivo screening of a large number of physiologically still viable plants among a mutagenized population of *A. thaliana*. Moreover, the biochemical screen used was based on the accumulation of sterols not present in the control, thus leading to mutants deficient in activity of a sterol biosynthetic enzyme, such as *ste1*. This mutant still contained 31% of physiological Δ^5-sterols and accumulated 68% of Δ^7-sterols (*14*). It is interesting to observe that, in mutant *ste1*, to maintain a sufficient residual 5-DES activity, a mutation has emerged which limits decrease in the rate through matching weakening of transition-state binding by increasing K_m. In addition, it has been previously proposed that enzymes maximize rates by binding transition states strongly and substrate weakly (*31*), and that they should have evolved in this way (*32*). From this point of view, mutant T114S would be more highly evolved than the wild-type *A. thaliana* 5-DES.

How might Thr114 exert its effects on catalysis? A first proposal would give a role to threonine 114 in the formation of a hydrogen bond with the 3βOH group of the sterol substrate. This has already been proposed to be important in lanosterol-14α-demethylase (CYP51) from *Candida albicans*. In that case, change of Thr 315 to Ala lowered the catalytic activity of the enzyme by 50% (*33*). The present data, indicating a similar variation in K_m value for mutant T114I and T114S, makes this possibility less likely, although serine at position 114 would also be able to form the above-mentioned hydrogen bond. Moreover, previous studies with cycloeucalenol-isomerase and SAM-cycloartenol-C24-methyl-transferase, two other late enzymes in the plant sterol

biosynthetic pathway, indicated the requirement of a C-3β-hydroxyl group of the sterol substrate for initial enzyme−substrate interaction and significant binding (*34*, *35*).

Several general features of the hydroxylation mechanism carried out by one class of non-heme iron monooxygenase, exemplified by the methane−monooxygenase hydroxylase (MMOH), are understood. The mechanistic pathway of this class of enzyme can be written in a manner that mimics the rebound mechanism postulated for cytochrome P-450 during the incorporation of oxygen atoms from the dioxygen (*36*), although several other pathways following the initial C−H bond attack have been proposed (*37*). Recently, it was directly demonstrated in the case of MMOH that protons are required for the O−O bond cleavage steps of the catalytic cycle and that in each step a single proton is delivered by a single proton donor (*38*). Thr 213 in MMOH was suggested as a possible proton donor because it is closest to the diiron cluster and highly conserved among the oxygen-activating enzymes in both the Cyt P450 (*39*, *40*) and binuclear iron cluster monooxygenase families (*41*, *42*).

A possible role of Thr114 or Ser114 in catalysis would be to participate in the O−O bond cleavage step by delivery of a proton in one of the oxygen activation steps. In that case it would imply that the simple removal of the methyl group in the Thr114 residue leads to an important stabilization of the transition state of this step. Mutant desaturases with no threonine or serine might still function at a much slower rate by substituting protons derived from the solvent molecules. Further information will be needed to ascertain how Thr114 might exert its effects on catalysis. Nevertheless, a primary or secondary hydroxyl function at residue 114 is needed for maximal catalytic rate of the desaturase.

The data obtained in this study demonstrate that the 5-DES possesses eight high-consensus histidine residues which are essential for 5-DES activity. As discussed above, one possible function of these histidines could be to provide the ligands for a presumed catalytic Fe center. However, further experiments will be needed to ascertain the presence of iron in the 5-DES and the precise structural role of these histidine-rich motifs.

Site-directed mutation of seven other conserved residues led to important kinetic variations in the desaturase, but these residues, in contrast to the eight histidines, were not essential for 5-DES activity. In absence of structural information for this class of membrane-bound desaturases, we do not make any hypothesis concerning the structural localization of this latter group of mutated residues, except for the abovementioned mutation at position 114. One could ask whether some of these latter mutated desaturases might interact differently with yeast cytochrome b_5. It is difficult to answer this question directly since, to the best of our knowledge, we described herein the first in vitro functional yeast recombinant membrane-bound lipid desaturation system and data in the literature are also based upon heterologous systems. For example, rat liver wild-type and mutant cytochrome b_5 proteins have recently been functionally characterized using the yeast cytochrome b_5 reduction system (*42*). The results concerning a number of mutated 5-DESs show the limits of a method consisting of choosing evolutionarily conserved residues to identify catalytically essential residues based solely on the molecular evolution of a group of enzymes. On the other hand, data obtained with the in vivo selected mutation at position 114, affecting a functionally conserved residue, led to more important information about the structure/function of the protein. In a number of cases, such in vivo selected mutations have led to important new functional properties of the protein studied (*43*). Finally, it should be pointed out that the data obtained herein with the heterologously expressed mutant desaturase T114I agree fully with those obtained previously for this mutant, in vivo, in *A. thaliana* (*9*).

In conclusion, the use of both in vivo and in vitro assays to analyze the activity and kinetics of the first series of mutant Δ^7-sterol-C5(6)-desaturases in this work has opened a door to our understanding of the structure−function relationship of this membrane-bound desaturase. The precise mechanism of the catalytic reaction carried out by the 5-DES, as well as that of the parent plant sterol-4α-methyl oxidase (*44*), and the involvement of the active-site residues will need further studies. The present work also demonstrates that it is possible to improve by site-directed mutagenesis the activity of an enzyme involved in the postsqualene segment of sterol biosynthesis.

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