Eight Histidine Residues Are Catalytically Essential in a Membrane-Associated Iron Enzyme, Stearoyl-CoA Desaturase, and Are Conserved in Alkane Hydroxylase and Xylene Monooxygenase†

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ABSTRACT: The eukaryotic fatty acid desaturases are iron-containing enzymes that catalyze the NAD(P)H- and O_2-dependent introduction of double bonds into methylene-interrupted fatty acyl chains. Examination of deduced amino acid sequences for the membrane desaturases from mammals, fungi, insects, higher plants, and cyanobacteria has revealed three regions of conserved primary sequence containing $HX_{(3\text{ or }4)}H$, $HX_{(2\text{ or }3)}HH$, and $HX_{(2\text{ or }3)}HH$. This motif is also present in the bacterial membrane enzymes alkane hydroxylase (ω-hydroxylase) and xylene monooxygenase. Hydropathy analyses indicate that these enzymes contain up to three long hydrophobic domains which would be long enough to span the membrane bilayer twice. The conserved His-containing regions have a consistent positioning with respect to these potential membrane spanning domains. Taken together, these observations suggest that the membrane fatty acid desaturases and hydrocarbon hydroxylases have a related protein fold, possibly arising from a common ancestral origin. In order to examine the functional role of these conserved His residues, we have made use of the ability of the rat Δ^9 desaturase gene to complement a yeast strain deficient in the Δ^9 desaturase gene function (*ole1*). By site-directed mutagenesis, eight conserved His residues in the rat Δ^9 desaturase were individually converted to Ala. Each His → Ala mutation failed to complement the yeast *ole1* mutant. In contrast, mutation of three nonconserved flanking His residues or a partially conserved Arg residue within the conserved motif to Ala allowed for complementation of the *ole1* phenotype. Western blot analysis showed that steady-state expression levels were equivalent for the wild-type rat desaturase and for all mutants, suggesting that the conserved His residues are essential for catalytic function. One role for these His residues would be to act as ligands for the iron atom(s) contained in these enzymes. On the basis of these results and the presently available spectroscopic data, we recognize the possibility that the membrane desaturases, alkane hydroxylase, and xylene monooxygenase contain a new structural type of diiron center.

In all eukaryotes, fatty acids are desaturated by a number of different NAD(P)H and O_2-dependent multiprotein enzyme complexes (Fulco, 1974). Both soluble and membrane-associated enzymes have been described. Despite this structural difference, the soluble and membrane desaturases have several important similarities including the catalytic requirement for iron (Nagai & Bloch, 1968), the inhibition by metal chelators (Jaworski & Stumpf, 1974), the stereospecificity of the desaturation reaction (Schroepfer & Bloch, 1965), and the kinetic isotope effects observed for C–H bond cleavage (Morris, 1970). These similarities suggest a common mechanism is used for the desaturation reaction, which may include the requirement for a structurally related catalytic active site.

Recently, the soluble stearoyl-acyl carrier protein (stearoyl-ACP) Δ^9 desaturase (Δ9D)[1] has been cloned and expressed from plants (Shanklin & Somerville, 1991; Thompson et al., 1991). This enzyme introduces a *cis* double bond at the 9,10 position of stearoyl-ACP to form olelyl-ACP. Through the use of optical and Mössbauer spectroscopies, we have shown that the soluble desaturase is one of an increasing number of proteins containing oxo or hydroxo-bridged diiron clusters (diiron-oxo proteins) (Fox et al., 1993). In the preceding paper in this issue, we demonstrate that these proteins can be divided into two evolutionarily *unrelated* classes with the respiratory protein, hemerythrin, being a prime example of class I, and the soluble desaturase being a member of class II (Fox et al., 1994). Two structurally characterized members of class II, the iron-containing component of ribonucleotide reductase (R2) (Nordlund & Eklund, 1993) and the hydroxylase component of methane monooxygenase (MMOH) (Rosenzweig et al., 1993), use these clusters to catalyze diverse reactions requiring O_2-activation chemistry (Sanders-Loehr, 1988). On the basis of the conserved primary structural motif in R2, the bacterial hydrocarbon hydroxylases, and the stearoyl-ACP desaturases, we have proposed a model for the active site of the soluble desaturases which includes a partial assignment of the cluster ligands.

None of the membrane desaturases sequenced to date contain the iron binding motif found in the soluble desaturases (Yadav et al., 1993; Thiede et al., 1986; Stukey et al., 1989). However, all membrane desaturases, from a wide distribution

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[1] Abbreviations: ACP, acyl carrier protein; Δ9D, stearoyl-ACP Δ^9 desaturase; MMOH, methane monooxygenase hydroxylase component; R2, iron-containing component of ribonucleotide reductase.

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Table 1: Sequences of the Oligonucleotide Primers Used to Construct Site-Directed Mutants of the Rat Δ^9 Desaturase Gene[a]

flanking primer 1	mutation	strand	mutagenic oligonucleotide	site	flanking primer 2	PDs3-358 fragment
rat623	H119A	+	CACAGCCGGGGCCGCGCGCTTGTGG	*Acc*II	M13R	*Eco*RV *Nco*I
rat623	H124A	+	CGCTTCTGGAGCGCGAGGACTTAC	*Acc*II	M13R	*Eco*RV *Nco*I
rat401	H156A	–	CGCCCGGGATGCCCGCGCCC	none	M13F	*Nco*I *Sac*I
rat401	R157N	–	GTTGTGATCCCGGGCCCATTC	none	M13F	*Nco*I *Sac*I
rat401	H159A	–	ACTTGTGGGCGGCGCGGT	none	M13F	*Nco*I *Sac*I
rat401	H160A	–	AGAACTTGGCGTGGGCGC	none	M13F	*Nco*I *Sac*I
rat401	H166A	–	GGTCGGCGGCTGTCTCAG	none	M13F	*Nco*I *Sac*I
rat401	H170A	–	GGGAGTTGGCAGGGTCGG	none	M13F	*Nco*I *Sac*I
M13F	H297A	+	GGGCGAGGGCTTCGCGAACTAACCAT	*Bst*UI	M13R	*Nco*I *Sac*I
M13F	H300A	+	TCCACAACTACGCGCACGCCTTC	*Bst*UI	M13R	*Nco*I *Sac*I
M13F	H301A	+	TCCACAACTACCATGCCGCGTTCCCC	*Bst*UI	M13R	*Nco*I *Sac*I
M13F	H315A	+	ACCGCTGGGCCATCAACTTCACC	*Hae*III	M13R	*Nco*I *Sac*I
sequences of flanking primers						
rat401		+	GGATCTTCCTCATCATTGCC			
rat623		–	CCAGCTTCTCAGCTTTCAGGTCA			
M13F		–	CGCCAGGGTTTTCCCAGTCACGAC			
M13R		+	AGCGGATAACAATTTCACACAGGA			

[a] Flanking primers 1 were used in conjunction with the corresponding mutagenic oligonucleotide to amplify a portion of the rat Δ^9 desaturase open reading frame containing site-directed mutations as described under Material and Methods. The sequences of the nonmutagenic oligonucleotides used as flanking primers are indicated at the bottom of the table. The strand designation + refers to the coding strand; – refers to the noncoding strand. Sequences of the mutagenic oligonucleotides are reported in the 5′ to 3′ direction.

of organisms, contain three regions of conserved His-containing primary sequence. Surprisingly, the bacterial membrane enzymes alkane hydroxylase (Kok et al., 1989) and xylene monooxygenase (Suzuki et al., 1991) also have His-containing primary sequences that are shown here to be closely related to those in the membrane desaturases.

As part of our ongoing efforts to understand the structural and catalytic properties of the eukaryotic fatty acid desaturases, we have probed the role of these His residues by study of the best characterized membrane desaturase, the stearoyl-CoA Δ^9 desaturase from rat liver (Strittmatter et al., 1974). This enzyme introduces a *cis* double bond at the 9,10 position of stearoyl-CoA to form oleyl-CoA. The rat Δ^9 desaturase has been purified and the corresponding cDNA has been isolated (Thiede et al., 1986). Expression of the rat Δ^9 desaturase gene in a Δ^9 desaturase-deficient mutant of *Saccharomyces cerevisiae* (*ole1*; (Stukey et al., 1989) allows for complementation of the mutation (Stukey et al., 1990). This complementation has thus provided a method to assess the functional significance of the conserved His residues in the membrane desaturases.

MATERIALS AND METHODS

Site-Directed Mutagenesis. The procedures used for cloning and plasmid manipulations were as previously described (Maniatis et al., 1982). The coding region of pDs3-358 was mutated using the polymerase chain reaction so that selected His residues were replaced by Ala. Table 1 shows the flanking primers and mutagenic oligonucleotides used to introduce the appropriate site-directed mutations. Where feasible, mutagenic oligonucleotides were also designed with a silent mutation encoding a novel restriction site (indicated in Table 1) to facilitate distinction of mutant and wild-type fragments at subsequent cloning steps. The strategy used for mutation and expression of the rat Δ^9 desaturase gene pDs3-358 is outlined in Figure 1. Flanking primer 1 (Table 1) was used in conjunction with the mutagenic oligonucleotides (Table 1) to amplify a region of pDs3-358 (round 1 product of Figure 1). The round 1 product was gel purified and then used as a primer along with flanking primer 2 (Table 2, corresponding to a pUC8 flanking region) in a second round of polymerase chain reaction to amplify the desaturase from pDs3-358 (round 2 product of Figure 1). The round 1 product encodes a region of the rat Δ^9 desaturase and approximately 30 bp of flanking pUC8 sequence. The product 2 fragment was cloned into the vector pCRII (Invitrogen) and sequenced using the dideoxy chain termination method (Sanger et al., 1977) to confirm the introduction of the mutation and to ensure that secondary mutations had not been introduced. The *Nco*I–*Sac*I fragment containing the mutated portion of the cDNA was released from the pCRII vector by restriction digestion and introduced into pDs3-358. The presence of the mutated fragment was confirmed by either restriction analysis or resequencing. The *Bam*HI–*Sac*I fragment encoding the entire rat open reading frame was then excised from pDs3-358 and introduced into the corresponding restriction sites in the yeast expression plasmid YEp 352 YOPR containing the yeast OLE1 promotor and 27 N-terminal codons of the *OLE1* gene (Stukey et al., 1990). This plasmid was amplified in *E. coli* DH5αF′ (Gibco BRL Research Labs) and transformed into the *ole1* yeast disruptant strain L8-14C (α, oleΔ1::LEU2, ura3-52, his4) to uracil prototrophy by electroporation using an Electrocel manipulator 600 (BTX, Inc.).

Complementation Testing. Yeast strain L8-14C containing the wild-type rat Δ^9 desaturase or the site-directed mutations was plated on synthetic defined media (0.7% yeast nitrogen base minus amino acids, 2% dextrose, 0.005% histidine, 1% Tergitol NP40, 0.2% casamino acids, 2% agar) either lacking unsaturated fatty acids or supplemented with 0.5 mM oleic and 0.5 mM palmitoleic acids. Plates were incubated at 30 °C for 4 days before analysis.

Denaturing Gel Electrophoresis and Western Blot Analysis. Yeast strain L8-14C containing the wild-type or mutant rat desaturase was grown in media supplemented with unsaturated fatty acids to 2×10^7 cells/mL and transferred to a medium lacking unsaturated fatty acids for 8 h. Cells were collected by centrifugation, washed once with water, and disintegrated in sample buffer (Laemmli, 1970) by vortexing with glass beads. Proteins were separated by denaturing gel electrophoresis and transferred to nitrocellulose for western blotting (Towbin et al., 1979). The western blot was probed with rabbit anti-rat stearoyl-CoA desaturase antibodies using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as detection substrates.

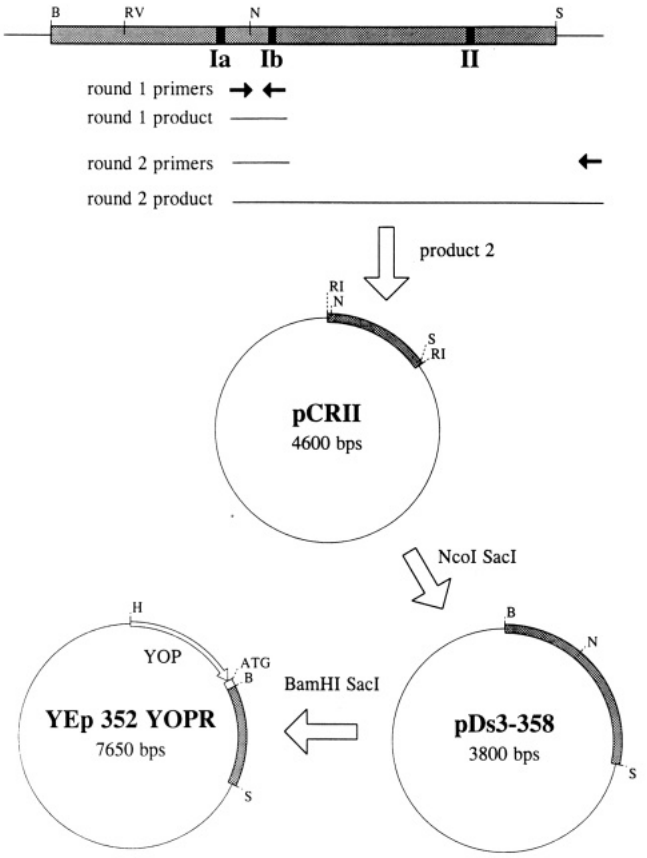

FIGURE 1: Strategy for construction of the site directed mutants of the rat Δ^9 desaturase and transfer of the mutated gene to yeast expression plasmid YEp 352 YOPR as described under Materials and Methods. Restriction site codes: B, *Bam*HI; RV, *Eco*RV; N, *Nco*I; S, *Sac*I; RI, *Eco*RI; H, *Hin*dIII.

Computer Sequence Analysis. Derived amino acid sequences were obtained from GenBank and compared using FASTP (Pearson & Lipman, 1988) and BESTFIT (Needleman & Wunsch, 1970). Search algorithms for location of His-containing sequences were developed by the genome research group at Brookhaven National Laboratory. Domain analysis was performed using the PROSIS computer program employing the Kyte and Doolitle algorithm (Kyte & Doolittle, 1982). Protein parsimony algorithms were obtained as part of the Phylogeny Inference Package (Felsenstein, 1989).

RESULTS AND DISCUSSION

Membrane desaturases are present in a diverse group of organisms, including mammals, fungi, insects, higher plants, and cyanobacteria. Table 2 lists representative desaturases that insert double bonds into the 6, 9, 12, and 15 (ω-3) positions of various C_{18} fatty acid derivatives. These enzymes accept reducing equivalents from different electron donors including cytochrome b_5 and [2Fe 2S] ferredoxin (Strittmatter et al., 1974; Wada et al., 1993). Except for the stearoyl-ACP Δ^9 (Nagai & Bloch, 1968) and coriander Δ^4 (Cahoon et al., 1992) desaturases, all desaturases are integral membrane proteins, which has significantly hindered efforts to purify and study these enzymes. Consequently, the nature of the iron-containing active site in the membrane desaturases is poorly defined. Nevertheless, the stearoyl-CoA desaturase from rat liver, the one membrane desaturase which has been purified, does exhibit an absorption spectrum (Strittmatter et al., 1974)

Table 2: Comparison of Source Organisms, Substrates, Reactions, and Electron Donors for the Membrane-Associated Desaturases and Hydroxylases[a]

abbreviation	source	substrate	electron donor
	Membrane Desaturases		
Δ^9 position			
rat	mammalian liver	18:0-CoA	cytochrome b_5
yeast	fungal endoplasmic reticulum	18:0-CoA	cytochrome b_5
mouse	mammalian liver	18:0-CoA	cytochrome b_5
tick	insect salivary gland	18:0-CoA	cytochrome b_5
Δ^{15} position (ω-3)			
Atfad7	plant chloroplast	18:2-MGDG	ferredoxin
Bnfadd	plant chloroplast	18:2-MGDG	ferredoxin
Gsoja	plant chloroplast	18:2-MGDG	ferredoxin
Rcfad7	plant plastid	18:2-MGDG	ferredoxin
Bnfad3	plant endoplasmic reticulum	18:2-PC	cytochrome b_5
Δ^{12} position			
Atfad2	plant endoplasmic reticulum	18:1-PC	cytochrome b_5
SyndesA	cyanobacteria	18:1-MGDG	ferredoxin
Δ^6 position			
Synd6	cyanobacteria	18:2-MGDG	ferredoxin
	Membrane Hydroxylases		
alkB	eubacteria	*n*-octane	rubredoxin
xylM	eubacteria	xylene	xylA

[a] Rat, *Rattus norvegicus* Δ^9 desaturase (Thiede et al., 1986); yeast, *S. cerevisiae* Δ^9 desaturase (Stukey et al., 1990); mouse, *Mus musculus* Δ^9 desaturase (Ntambi et al., 1988); tick, *Amblyomma americanum* Δ^9 desaturase (Luo, 1993); Atfad7, *Arabidopsis thaliana* chloroplast Δ^{15} desaturase; Bnfadd3, *Brassica napus* chloroplast Δ^{15} desaturase and Gsoja, *Glycine soja* chloroplast Δ^{15} desaturase (Yadav et al., 1993); Rcfad7, *Ricinus communis* plastid Δ^{15} desaturase (Iba et al., 1993); Atfad2, *Arabidopsis thaliana* endoplasmic reticulum Δ^{12} desaturase (Okuley et al., 1994); Bnfad3, *Brassica napus* endoplasmic reticulum Δ^{15} desaturase (Arondel et al., 1992); SyndesA, *Synechocystis* sp. strain PCC 6803 Δ^{12} desaturase (Wada et al., 1990); Synd6, *Synechocystis* sp. strain PCC 6803 Δ^6 desaturase (Reddy et al., 1993), alkB, *Pseudomonas oleovorans* alkane hydroxylase (Kok et al., 1989) and; xylM, *Pseudomonas putida* xylene monooxygenase (Suzuki et al., 1991). The substrates indicated are the principal substrates; each enzyme will use alternate substrates at various efficiencies. Substrate abbreviations: CoA, coenzyme A; PC, phosphatidylcholine; MGDG, monogalactosyl diacylglycerol.

that is characteristic of an oxo-bridged diiron cluster (Sanders-Loehr, 1989).

Figures 2 and 3 contain portions of primary sequence for the membrane desaturases listed in Table 2. A high degree of sequence identity (greater than 85%) is observed for the Δ^{15} desaturases from plants (Yadav et al., 1993), whereas relatively lower sequence identity is observed for the desaturases with other positional specificities (e.g., the Δ^9 desaturases listed in Figures 2 and 3 have between 8 and 85% identity for mammalian, fungal, and insect enzymes). Pairwise comparison of the desaturases with different positional specificities also reveals low sequence identity (typically less than 25%). However, the regions shown in Figures 2 and 3 have dispersed homology between all compared sequences, including desaturases with different positional specificities.

Conserved Sequence Motifs in the Membrane Desaturases and Hydroxylases. Recently, we have demonstrated the presence of an oxo-bridged diiron cluster in the soluble plant stearoyl-ACP desaturase (Δ9D) from its characteristic absorption, Mössbauer and resonance Raman spectroscopic properties (Fox et al., 1993, 1994). In the preceding paper (Fox et al., 1994), we have also presented evidence for primary sequence identity between the soluble stearoyl-ACP desaturases [stearoyl-ACP Δ^9 desaturase and coriander Δ^4 desaturase (Cahoon et al., 1992)] and other class II diiron proteins: the R2 component of ribonucleotide reductase and the soluble

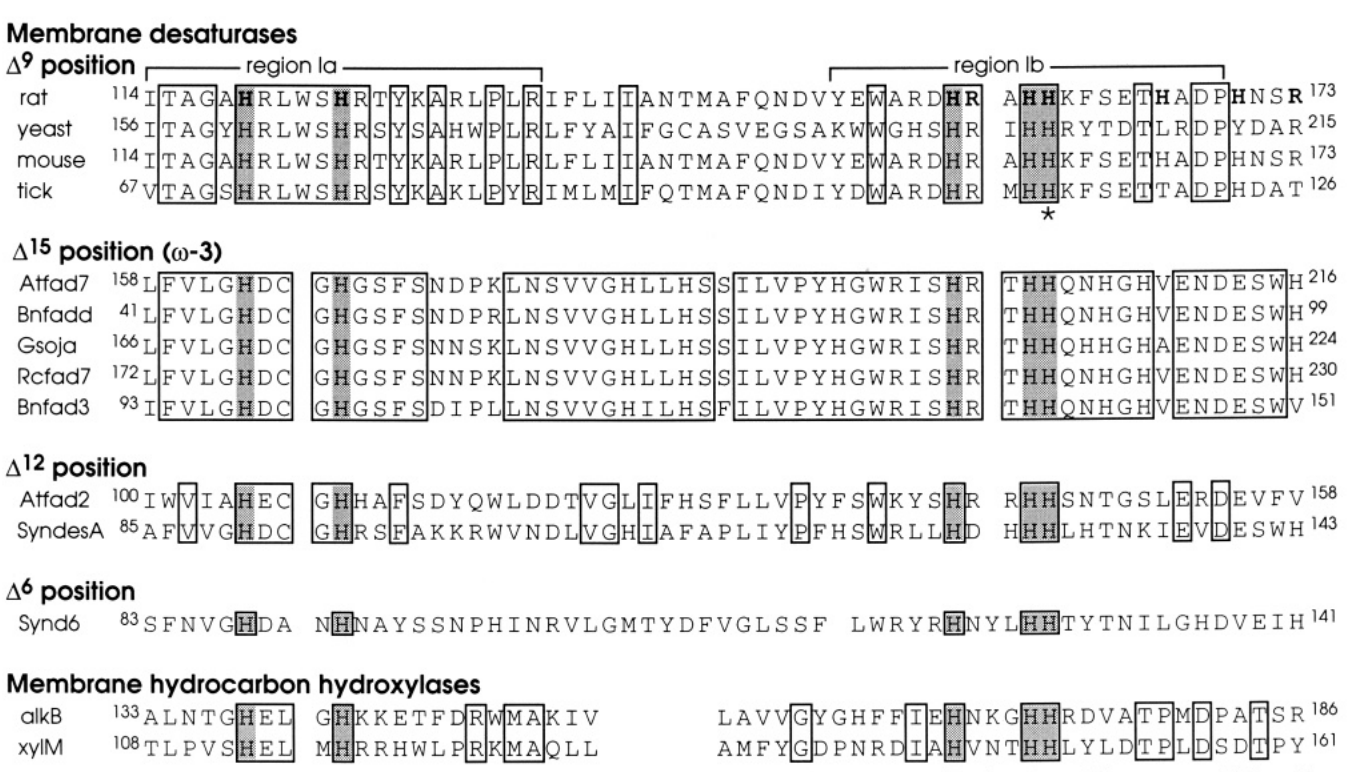

FIGURE 2: Comparison of the region Ia and Ib amino acid sequences of the membrane desaturases and hydroxylases. Five conserved His residues are indicated by gray shading (two in region Ia, three in region Ib). The contiguous primary sequences were aligned relative to the His residue marked by an asterisk. Residues changed by site-directed mutagenesis are indicated by bold type. Positions containing identically conserved residues are enclosed in boxes. Gaps were introduced to facilitate sequence alignments. Abbreviations are as described in Table 2.

FIGURE 3: Comparison of the region II amino acid sequences of the membrane desaturases and hydroxylases. Three conserved His residues are indicated by gray shading. Other details are provided in the caption to Figure 2.

bacterial hydrocarbon hydroxylases (MMOH, toluene-4-monooxygenase, and phenol hydroxylase). With reference to the X-ray structures of R2 (Nordlund & Eklund, 1993) and MMOH (Rosenzweig et al., 1993), these alignments reveal a consensus iron binding motif containing two repeats of EX_2H separated by approximately 100 amino acids. This iron-binding motif provides four of the six protein-derived ligands to the diiron cluster. An additional Glu residue which acts as a monodentate ligand to one cluster iron also appears to be conserved among all of these proteins.

None of the membrane desaturases sequenced to date contain the active site motif observed for the soluble desaturases. However, a previously published study has shown that the rat and yeast Δ^9 stearoyl-CoA desaturases contain three regions

of conserved His-containing primary sequence (Stukey et al., 1990). Figures 2 and 3 show that the Δ^9 desaturases from mouse and tick and the Δ^6, Δ^{12}, and Δ^{15} desaturases from higher plants and cyanobacteria have *the same* three regions of primary sequence containing conserved His residues. For reference, the three regions have been labeled Ia, Ib, and II in Figures 2 and 3, and the conserved His residues are indicated by gray shading. In region Ia (Figure 2), all Δ^9 desaturases contain the sequence HX_4H, while the plant and cyanobacterial desaturases contain the sequence HX_3H. In region Ib (Figure 2), 11 of the 12 representative desaturases contain the sequence HX_2HH, while the Δ^6 desaturase from the cyanobacterium *Synechocystis* sp. strain PCC 6803 has HX_3HH. In region II (Figure 3), the same 11 representative desaturases have a second occurrence of the sequence HX_2HH, while the *Synechocystis* sp. strain PCC 6803 Δ^6 desaturase has a second occurrence of HX_3HH.

The structural relationship between the soluble stearoyl-ACP desaturases and the bacterial hydroxylases (MMOH, toluene-4-monooxygenase, and phenol hydroxylase) reported in the preceding paper suggested that a similar structural relationship may occur between the membrane desaturases and other membrane enzymes. Specifically, we have focused on the properties of the alkane hydroxylase from *Pseudomonas oleovorans* (Kok et al., 1989; van Beilen et al., 1992) and xylene monooxygenase from *Pseudomonas putida* (Suzuki et al., 1991), which are two relatively well-characterized bacterial membrane hydroxylases. The preferred substrates and electron donors for these hydroxylases are listed in Table 2. Previous studies have shown that alkane hydroxylase and xylene monooxygenase are closely related proteins that have three regions of conserved His-containing primary sequence (van Beilen et al., 1992; Suzuki et al., 1991). Figures 2 and 3 show that these three His-containing regions appear to be closely related to the His-containing sequences observed in the membrane desaturases, both in primary sequence and in overall spacing.

To address the potential significance of these His-containing sequences, we have searched the protein databank to determine how frequently such motifs occur (GenBank release 82.0, April, 1994, containing 95 243 entries). Inspection of the aligned sequences in Figures 2 and 3 reveals the following consensus sequence: $HX_{(3\text{ or }4)}HX_{(20-50)}HX_{(2\text{ or }3)}HHX_{(100-200)}HX_{(2\text{ or }3)}HH$. Searches using this consensus sequence provided only 20 matches (after discarding proteins containing poly-His sequences), which comprise 0.02% of proteins, demonstrating this His-containing motif clearly occurs infrequently. Among the 20 proteins identified to contain this motif, 17 are fatty acid desaturases, two are hydrocarbon hydroxylases (alkane hydroxylase and xylene monooxygenase), and one is a hemoglobin from *Pseudoterranova decipiens*. Thus, of the 20 identified proteins, 19 represent two types of enzymes that are proposed to both have iron-containing active sites and to require O_2 activation as part of the catalytic cycle.

Hydropathy Analysis. Previously, Martin and co-workers (Stukey et al., 1990) had compared the hydropathy of the rat and yeast desaturases and proposed a model in which each protein had two long hydrophobic domains, each capable of spanning the membrane twice, such that the three hydrophilic domains (containing the conserved His residues) reside on the cytoplasmic face of the endoplasmic reticulum membrane. A diagram of this arrangement is shown in Figure 4A. A structural model has been presented for the alkane hydroxylase (van Beilen et al., 1992), whose sequence contains three similar hydrophobic domains, each capable of crossing the membrane

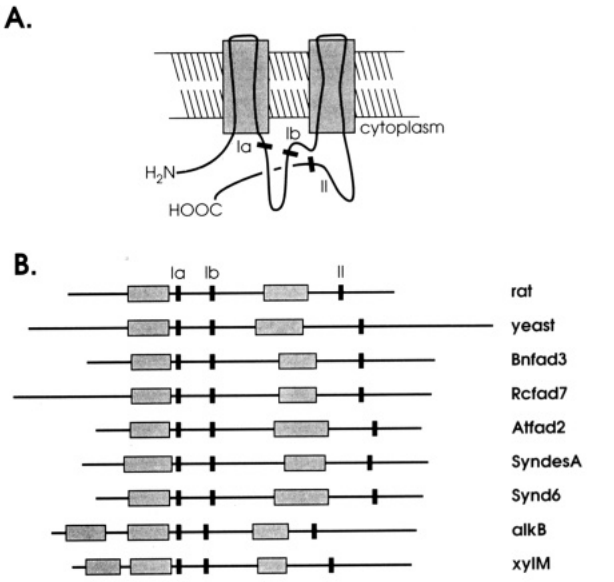

FIGURE 4: (A) Model of the topology of the desaturase sequences shown in panel B. (B) Hydrophobic domain structure of the membrane desaturases and hydroxylases. Gray boxes represent hydrophobic domains containing greater than 40 amino acid residues, capable of spanning the membrane twice. The location of His-containing regions Ia, Ib, and II are indicated by solid boxes. All sequences are aligned relative to the conserved His residues in region Ia. The hydroxylases have an additional N-terminal domain capable of spanning the membrane twice. Abbreviations are as described in Table 2.

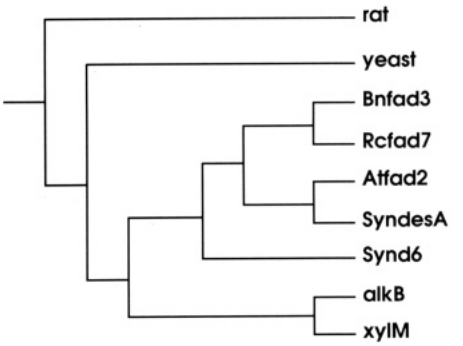

FIGURE 5: Phylogenic relationship between the membrane desaturases and hydroxylases as determined by parsimony analysis (Felsenstein, 1989). Regions I and II were combined for the analysis. Abbreviations for various gene products are described in Table 2.

twice. Experimental support for multiple membrane-spanning domains was provided by making fusions of the alkane hydroxylase with either β-galactosidase or alkaline phosphatase at various positions in the primary sequence. The pattern of catalytic activity observed for these fusion proteins indicate that the alkane hydroxylase has three domains that span the periplasmic membrane twice and four hydrophilic domains that face into the cytoplasm. We have performed hydropathy analysis on seven of the 11 desaturase sequences shown in Figures 2 and 3 and have obtained results consistent with this structural model for all compared sequences. A schematic representation of this hydropathy analysis is presented in Figure 4B, along with the positions of the His-containing regions. For both the desaturases and hydroxylases, the conserved His-containing regions are uniformly predicted to be in hydrophilic regions of primary sequence, rather than in membrane spanning domains, and each of these domains is predicted to occur on the cytoplasmic face of the membrane. Moreover, it is also notable that the distance between the His-containing regions and the end of the previous hydrophobic domain is highly conserved in all of the compared sequences:

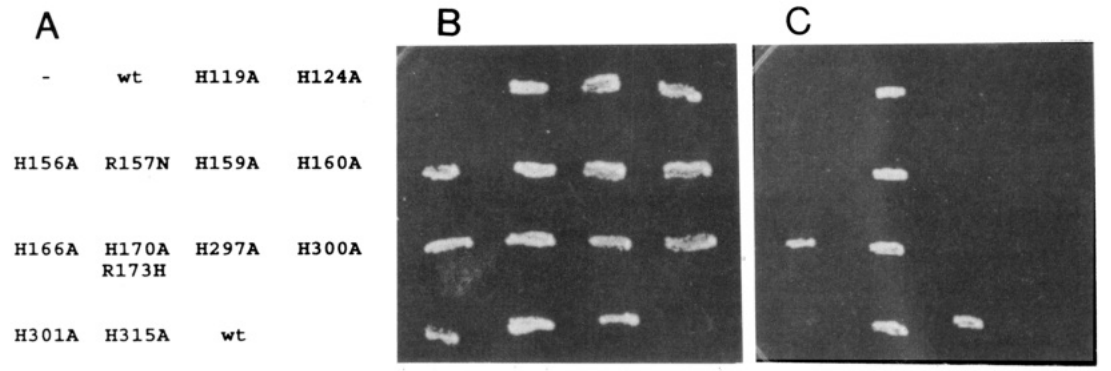

FIGURE 6: Growth of yeast *ole1* mutant strain L8-14C lacking or containing either the wild-type rat Δ^9 desaturase or mutant forms of the gene. (A) Constructs found in panels B and C. (B) Strain L8-14C plated onto media containing unsaturated fatty acids. (C) Strain L8-14C plated onto media lacking unsaturated fatty acids.

for region Ia, within 10 residues; for region Ib, between 37 and 44 residues; and for region II, between 29 and 52 residues. Since all of the substrates for these enzymes are highly hydrophobic (Table 2), they will likely partition into the lipid bilayer. In contrast, the electron donors for these enzymes are either soluble or peripheral membrane proteins (Table 2; Strittmatter et al., 1974; Peterson & Coon, 1968; Wada et al., 1993). These two properties suggest that an active site assembled from these His-containing sequences may occur at or near to the membrane surface (Figure 4A). Thus, on the basis of the conserved His sequences, the similar hydropathy characteristics, and on the potential evolutionary relationship, we propose that the membrane desaturases and hydroxylases can all be placed within a single structural category.

Phylogenic Relationships. The shared sequence homology in regions I and II, the conserved His residues, and the conserved structural organization with respect to potential membrane spanning domains suggest that the membrane desaturases and hydroxylases may share a common ancestral origin. Using parsimony analysis software that takes into account the number of mutations required to transform one amino acid sequence into another, we have derived a phylogenic tree (Figure 5) representing the evolutionary relationship between these genes (Felsenstein, 1989). This analysis suggests that the membrane desaturases and hydroxylases represent a gene family which has evolved from a prototype most closely resembling the rat Δ^9 desaturase gene. It is reasonable that a Δ^9 desaturase gene is most similar to the ancestral gene because the 9,10 position is first to be desaturated in eukaryotes and this monounsaturated fatty acid then acts as a substrate for subsequent desaturation reactions leading to polyunsaturated fatty acids (Holloway, 1983).

Site-Directed Mutagenesis and Complementation Studies. In order to further investigate the role of the conserved His residues in the rat desaturase, we have made use of the ability of the rat Δ^9 gene to complement the *ole1* mutation in *S. cerevisiae* strain L8-14C (Stukey et al., 1990). By using a polymerase chain reaction-based site-directed mutagenesis strategy (Figure 1), eight conserved His residues were changed to Ala (indicated by bold type in Figures 2 and 3). In addition, three nonconserved His residues, two from flanking region Ib (H166 and H170) and one from flanking region II (H315), were changed to Ala. A translational fusion between the rat Δ^9 desaturase open reading frame containing these mutations and the promotor and the 5′ region of the yeast *OLE1* gene was then placed into the yeast expression plasmid YEp 352 YOPR and transferred to *ole1* mutant strain L8-14C. Strain L8-14C lacking the plasmid YEp 352 was unable to grow in the absence of uracil and unsaturated fatty acids, while all lines carrying the plasmid YEp 352 were viable on minimal plates supplemented with uracil and unsaturated fatty acids. In addition, strain L8-14C carrying the wild-type rat Δ^9 desaturase gene was viable on media lacking unsaturated fatty acids. Mutations in any of the eight conserved His residues prevented growth on minimal media lacking unsaturated fatty acids (Figure 6), whereas mutation in any of the three nonconserved His residues still permitted growth on minimal media lacking unsaturated fatty acids. Likewise, mutation of an Arg residue that is partially conserved among the mammalian and higher plant desaturases to Asn (Arg157 from region Ib in the rat Δ^9 desaturase, Figure 2) and the creation of a double mutation (H170A and R173H in the flanking region Ib, Figure 2) still permitted growth in the absence of unsaturated fatty acids.

In order to assess whether the His → Ala mutations had prevented growth on minimal media lacking unsaturated fatty acids *via* changes in mRNA or protein stability, immunoblots of total yeast cellular extracts were performed using anti-rat Δ^9 desaturase antibodies as a probe. Since cell extracts were made by introducing the yeast cells directly into the buffer used for denaturing electrophoresis, duplicate gels were run so that one could be stained for total protein content while the other could be immunostained for detection of the rat desaturase polypeptide. Figure 7A shows that the total protein content is essentially equivalent in all lanes, providing a control for the Western blotting experiments, and showing that the desaturase polypeptide is not abundantly overproduced. Yeast cultures lacking the rat Δ^9 desaturase gene have no immunoreactivity (Figure 7B, lane a), whereas all other lanes, containing either the wild-type rat Δ^9 desaturase gene or the site-directed mutant forms, show equivalent levels of immunostaining (Figure 7B, lanes b–n). Therefore, the nonviability of L8-14C carrying mutations in the conserved His residues of the Δ^9 desaturase gene can be attributed to a lack of function of the Δ^9 desaturase gene product rather than a lack of expression or a decrease in mRNA or protein stability.

Function of the Conserved His Residues. For the rat Δ^9 desaturase, mutation of any of eight conserved His residues results in a loss of desaturase activity, suggesting that these His residues may provide some essential catalytic functionality. Based on the propensity of His residues to serve as metal ligands, one possibility is that some or all of these His residues serve as iron ligands. Closely spaced arrangements of His residues are typical metal binding motifs in proteins: HH (Shapleigh et al., 1992); HXH in β strands; HX_2H in reverse turns; and HX_3H in α helices (Regan, 1993). The presence of closely spaced His residues allows for chelate binding modes

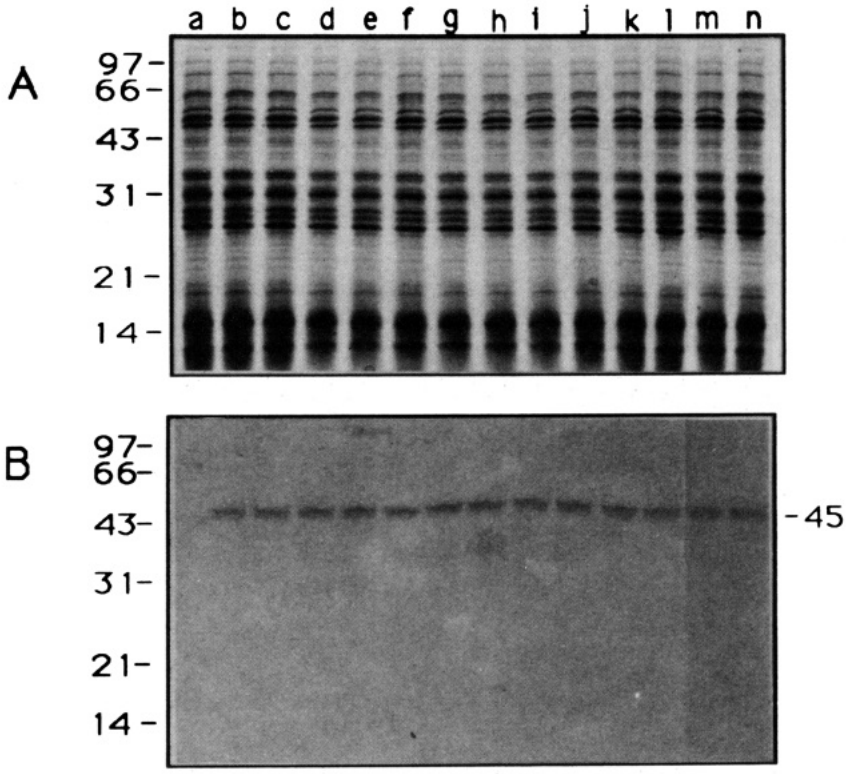

FIGURE 7: Denaturing polyacrylamide gel electrophoresis and Western blot analysis of yeast strain L8-14C lacking or containing either the wild-type rat Δ^9 desaturase or mutant forms of the gene. (A) Denaturing gel stained for total protein content with Coomassie Blue. (B) Denaturing gel immunostained after probing with antibodies directed against the rat Δ^9 desaturase. Relative molecular masses are indicated in kDa and the position of the 45-kDa yeast–rat desaturase fusion product is indicated in panel B. The lanes in panels A and B represent strain L8-14C containing the following rat Δ^9 desaturase constructs: (a) minus the rat Δ^9 desaturase gene; (b) wild-type rat Δ^9 desaturase gene; (c) H119A; (d) H124A; (e) H156A; (f) R157N; (g) H159A; (h) H160A; (i) H166A; (j) H170A/R173H; (k) H297A, (l) H300A; (m) H301A; (n) H315A.

that increase the affinity for transition metals by up to three orders of magnitude relative to a single His (Arnold & Haymore, 1991). Although α helices were originally associated with the HX_3H motif based on the X-ray structures of hemerythrin and hemocyanin (Holmes et al., 1991; Volbeda & Hol, 1989), α helices may also provide an HX_2H metal chelating motif as implied by the metal binding motif in the class II diiron-oxo proteins (Fox et al., 1994) or an HH metal chelating motif as seems likely for cytochrome *c* oxidase (Shapleigh et al., 1992). Thus, these results obtained on other His-ligated metalloproteins strongly support the assignment of the conserved $HX_{(3\text{ or }4)}H$, HX_2HH, and HX_3HH sequences in the membrane desaturases and hydroxylases as iron ligands. There are enough His residues in these conserved sequences to potentially act as ligands to two iron atoms. On the basis of this observation, and on the presently identified spectroscopic similarities (Strittmatter et al., 1974) with the soluble diiron-oxo proteins (Fox et al., 1994), we recognize the possibility that the membrane desaturases, alkane hydroxylase, and xylene monooxygenase contain a new type of diiron moeity, and thus constitute a third category of diiron proteins.

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