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A SINGLE AMINO ACID SUBSTITUTION CONVERTS CYTOCHROME $P450_{14DM}$ TO AN INACTIVE FORM, CYTOCHROME $P450_{SG1}$: COMPLETE PRIMARY STRUCTURES DEDUCED FROM CLONED DNAS

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Genes for lanosterol 14-demethylase, cytochrome $P450_{14DM}$, and a mutated inactive cytochrome $P450_{SG1}$ were cloned from S. cerevisiae strains D587 and SG1, respectively. A single nucleotide change resulting in substitution of Asp for Gly-310 of cytochrome $P450_{14DM}$ was found to have occured in cytochrome $P450_{SG1}$. In this protein the 6th ligand to heme iron is a histidine residue instead of a water molecule, which may be the ligand for the active cytochrome $P450_{14DM}$. Molecular models of the active sites of the cytochrome $P450_{14DM}$ and cytochrome $P450_{SG1}$ were built by computer modeling on the basis of the known structure of that of cytochrome $P450_{CAM}$ whose crystallographic data are available. The mechanisms which may cause a histidine residue to gain access to the heme iron are discussed.

A genetically altered cytochrome P450, $P450_{SG1}$ (1,2), which was derived from lanosterol 14-demethylase, $P450_{14DM}$ (3), has been isolated from a

Abbreviations: $P450_{14DM}$, lanosterol 14-demethylase from S. cerevisiae; $P450_{SG1}$, cytochrome P450 isolated from lanosterol 14-demethylase deficient mutant S. cerevisiae SG1; $P450_{CAM}$, camphor 5-exo-monooxygenase from Pseudomonas putida; $P450_{SCC}$, cholesterol side-chain cleavage cytochrome P450 from bovine adrenal; $P450_{b}$ and $P450_{c}$, rat liver microsomal cytochrome P450s; kbp, kilobase pairs.

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lanosterol 14-demethylation deficient yeast mutant, S. cerevisiae SG1 (ATCC 46786) (4,5). $P450_{SG1}$ showed no lanosterol 14-demethylase activity. Spectroscopic properties of $P450_{SG1}$ (6) were critically different from those of normal cytochrome P450s including $P450_{14DM}$, and indicated that the axial ligand trans to thiolate of $P450_{SG1}$ was a histidine residue (1,6), while that of the normal low-spin cytochrome P450 may be a water molecule (7,8,9,10). In spite of these differences, $P450_{SG1}$ and $P450_{14DM}$ were immunochemically identical and had identical peptide maps and 11 N-terminal amino acids (1). These observations suggested that the mutation giving rise to $P450_{SG1}$ caused local structural changes in the cytochrome which resulted in binding of a histidine residue to heme and loss of catalytic activity while retaining overall structural characteristics of $P450_{14DM}$. Accordingly, it was expected that comparison of the primary structure of $P450_{SG1}$ with that of $P450_{14DM}$ would provide some important information on the structure of the heme environment of $P450_{14DM}$.

The present communication describes primary structures of $P450_{SG1}$ and $P450_{14DM}$ deduced from the DNA sequences of their structural genes. The results indicated that only one amino acid substitution, i.e. Gly→Asp, occurred in $P450_{SG1}$. A possible structural change caused by this amino acid substitution is discussed, based on computer aided molecular modeling.

MATERIALS AND METHODS

Cultivation of Yeast. The nystatin-resistant yeast, S. cerevisiae SG1 and its nystatin-sensitive parent, S. cerevisiae D587, were cultivated as described previously (2).

Construction and Screening of Yeast Genomic Libraries. Yeast genomic DNAs isolated as described (11) were digested with BamHI and HindIII, fractionated by agarose gel electrophoresis, hybridized with a 17-mere oligonucleotide (complementary to nucleotides 1396 to 1422, Fig.2) designed according to Kalb et al. (12) and ^{32}P-labeled using T_4 polynucleotide kinase. A single positive band was observed at about 5kbp. Accordingly, BamH1-HindIII fragments of the genomic DNA of about 5kbp were prepared and inserted into pUC18 to construct a genomic library. The library was screened with the ^{32}P-labeled 17-mer oligonucleotide. Positive colonies were isolated and plasmid DNAs were purified as described (13).

Nucleotide Sequence Analysis. The HpaI-HindIII fragment or HpaI-DraI fragment (Fig. 1) of inserts were subcloned into M13mp18 or M13mp19. Sequencing was performed by the dideoxy chain terminator method (14,15) using chemically modified T_7DNA polymerase (16) (Sequenase™; United States Biochemical Corp.) Sequencing templates were prepared by deletion using T_4DNA polymerase (17) (International Biotechnologies, Inc.). Sequence data were analyzed by using the DNASIS software (Hitachi) and the GCG software package (18).

RESULTS

Isolation of genomic DNA clones for $P450_{SG1}$ and $P450_{14DM}$

Six and three clones out of about 1000 colonies from the genomic

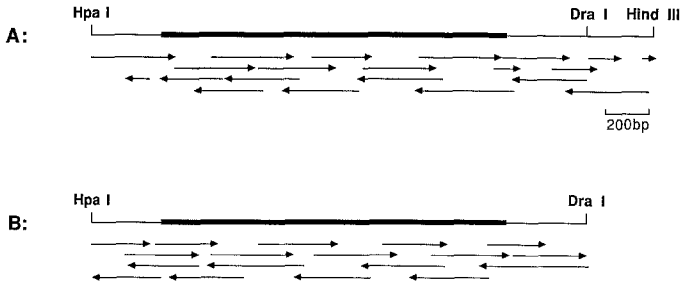

Fig.1 Sequencing strategy of $P450_{SG1}$ and $P450_{14DM}$ genes. Only those restriction enzymes relevant to the present work are shown. Thick and thin horizontal lines denote coding and noncoding regions, respectively. The direction and extent of sequence determination is shown by arrows. Sequencing was done as described in MATERIALS AND METHODS. A and B, $P450_{SG1}$ gene and $P450_{14DM}$ gene, respectively.

libraries of SG1 and D587, respectively, were found to be positive by colony hybridization analysis using the ^{32}P-labeled 17-mer oligonucleotide probe. Restriction map analysis of each of the clones showed that these clones were indistinguishable and we have chosen pgySG1.1 and pgy14DM.8 as representing clones for $P450_{SG1}$ and $P450_{14DM}$, respectively.

The HpaI-HindIII fragment and/or HpaI-DraI fragments of pgySG1.1 and pgy14DM.8 were submitted to nucleotide sequence analysis as described in MATERIALS AND METHODS (Fig. 1).

Mutation in $P450_{SG1}$

The DNA sequences and the deduced amino acid sequences of these genes are depicted in Fig. 2, where only residues different from those of $P450_{SG1}$ are shown for $P450_{14DM}$. The initiating methionines were easily identified by referring to the N-terminal amino acid sequences of $P450_{14DM}$ and $P450_{SG1}$ which had been determined on the purified enzymes (1). This sequence was identical in the two proteins and has been confirmed by the present studies.

Both P450 genes code for a protein of 530 amino acid residues. In the $P450_{SG1}$ gene, a single substitution of A for G in $P450_{14DM}$ gene was observed at nucleotide residue 929. This substitution resulted in an amino acid change from Gly to Asp at amino acid residue 310 which was located 53 amino acids upstream from the analogous tridecapeptide (Fig. 2) reported in many P450s (19). No other difference was found in the overlapping regions (nucleotides -332 to 1972) between these two P450s including 3'- and 5'-flanking regions so far determind.

Comparison between $P450_{14DM}$ genes in strains D587 and DBY939

When the sequence of the gene for $P450_{14DM}$ determined in the present study was compared to that determined by Kalb et al. (20) for the gene cloned from S. cerevisiae strain DBY939, several differences were noted (Fig. 2). In the overlapping sequences (-332 to 1972), two silent and one missense

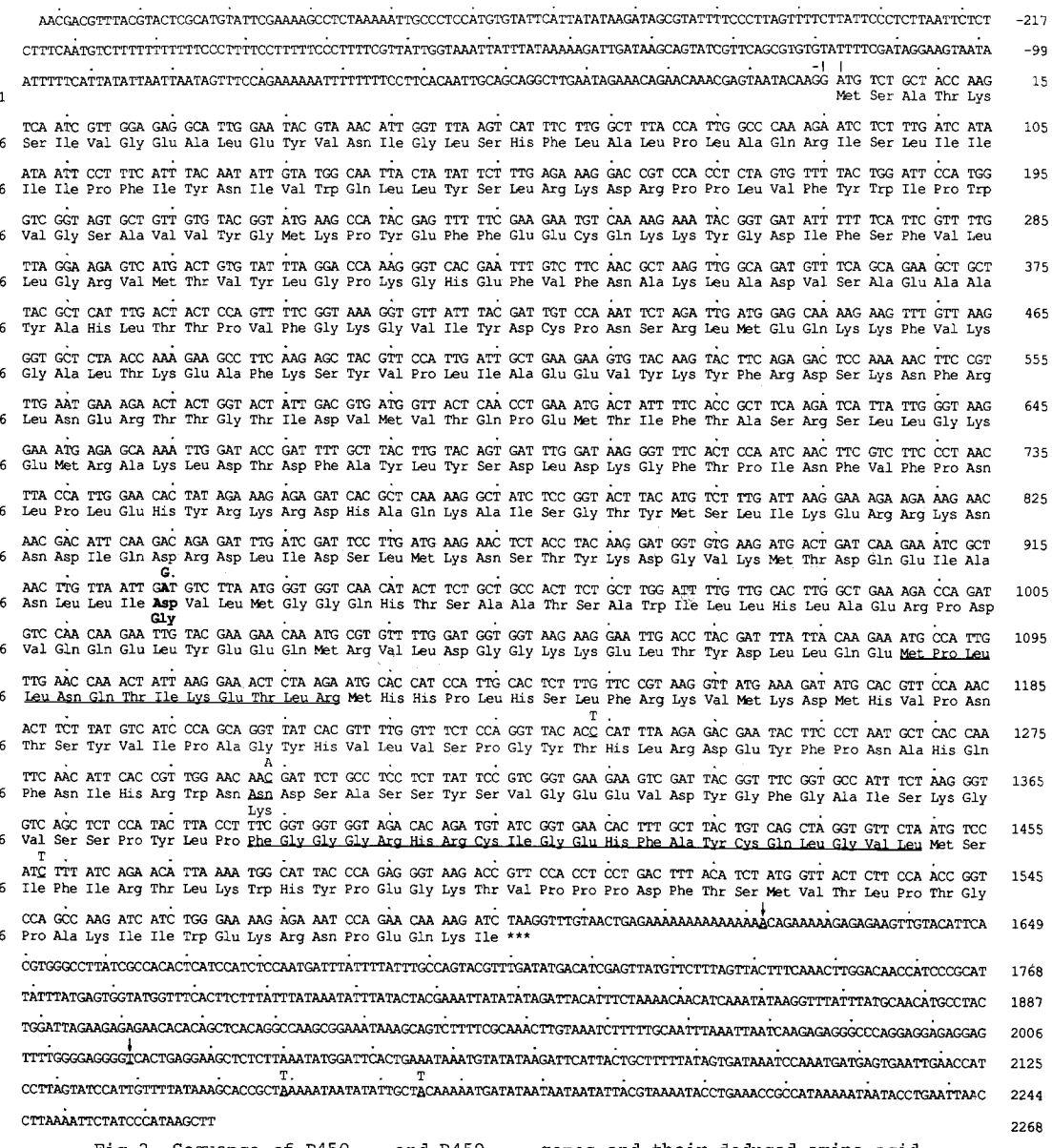

Fig.2 Sequence of $P450_{SG1}$ and $P450_{14DM}$ genes and their deduced amino acid sequences. Nucleotide residues are numbered in 5' to 3' direction, beginning with the first residue of the initiating methionine codon for $P450_{SG1}$. Amino acid residues deduced are expressed in three letter notation under nucleotide sequences and numbered beginning with the initiating methionine of SG1. The number of nucleotide and amino acid residues is given at the right and the left ends of each line, respectively. The sequence of the $P450_{14DM}$ gene of strain D587 was determined from -335 to 1972 corresponding to that of $P450_{SG1}$ gene. Nucleotides and the amino acids different from those of SG1 are shown in bold face letters. Residues in DBY939 gene (20) different from those of D587 and/or SG1 are shown above and below the underlined nucleotides or amino acids, respectively. Arrows indicate that the nucleotides are missing in DBY939 gene. Asterisks show the termination codon. The analogous tridecapeptides (19) and the HR2 region (23) are shown by long underlines.

substitutions were seen in the coding region and in 3'-flanking sequence, an extra A residue was observed for that of D587. As for the sequence

downstream of the nucleotide 1972, only comparison between those of SG1 and DBY939 was possible. However, if we take the comparison of the overlapping sequences of SG1, D587 and DBY939 into account, one insertion and two substitutions are likely to reflect also the difference between the sequences of strains D587 and DBY939.

Apparently, substitution of Asn-433 for Lys-433 in DBY939 has no serious effect on the enzyme activity of $P450_{14DM}$ (20).

DISCUSSION

$P450_{SG1}$ (1,2,6), from a lanosterol 14-demethylase deficient mutant, S. cerevisiae SG1, is an altered and inactive form of $P450_{14DM}$, lanosterol 14-demethylase (3,21). $P450_{SG1}$ is, however, immunochemically indistinguishable from $P450_{14DM}$; in addition, their peptide maps are identical (1). Hence $P450_{SG1}$ may be considered to be formed by a minor amino acid substitution in $P450_{14DM}$ which would not change overall conformation of the protein significantly. Actually, only one amino acid substitution was found between $P450_{SG1}$ and $P450_{14DM}$: Gly-310 of $P450_{14DM}$ was changed to Asp-310 in $P450_{SG1}$ (Fig. 2). As described previously, the most striking feature of $P450_{SG1}$ is the coordination of a histidine as the 6th ligand of the heme (1). Since $P450_{SG1}$ did not have newly introduced histidine, one of the pre-existing histidine residues must have gained access to the heme iron as the 6th ligand owing to substitution of Asp-310 for Gly-310. This observation suggested that the single amino acid substitution caused a significant local conformational change in the cytochrome, resulting in loss of activity. In contrast is the substitution in $P450_{DM}$ of yeast strain DBY939 of Lys-433 (20) for Asn-433, which was also residue 433 in $P450_{DM}$ of strain D587. Since DBY939 was not lanosterol 14-demethylase deficient (20), substitution of Asn-433 for Lys-433 or vice versa appears to show no essential effect on the activity of $P450_{14DM}$. In order to elucidate the structural basis for the shift of a histidine residue to the sixth ligand position of the heme resulting from an amino acid substitution, we have constructed a molecular model to compare the structure of the active site and its vicinity in $P450_{14DM}$ and $P450_{SG1}$.

By comparing with the sequences of distal helices in various P450s compiled by Poulos et al. (22), we assigned residues 309-325 of $P450_{14DM}$ as the distal helix sequence and aligned it with other sequences (Table 1). It is clear that the sequence has features common to the distal helices (22), e.g. residues 314-315 are Gly-Gly which are always Gly-Gly or Ala-Gly for the distal helices, residue 318 is Thr which is conserved in all P450s, and residues 311-316 form the hydrophobic stretch running over the distal heme

Table 1. Alignment of distal helices in various P450s

$P450_{CAM}$	G	L244	L	L	V	G	G	L	D251	T	V	V	N	F	L	S	F
$P450_{SCC}$	T	E321	M	L	A	G	G	V	N328	T	T	S	M	T	L	Q	W
P450b	L	S294	L	F	F	A	G	T	E301	T	T	S	S	T	T	L	R
P450c	F	D317	L	F	G	A	G	F	D324	T	I	T	T	A	I	S	W
$P450_{14DM}$	I	G310	V	L	M	G	G	Q	H317	T	S	A	A	T	S	A	W
$P450_{SG1}$	I	D310	V	L	M	G	G	Q	H317	T	S	A	A	T	S	A	W

surface (Table 1). Interestingly, the residues 309-325 contain the residue 310 which is the only residue different between $P450_{14DM}$ and $P450_{SG1}$, and a histidine residue at 317 (Table 1). A model of a part of the active site of $P450_{14DM}$ including the heme and the distal helix was built by using crystallographic data of $P450_{CAM}$ (22) registered in the Protein Data Bank (Fig. 3), and was used to analyze the probable structural changes caused by substitution of the residue 310.

In $P450_{14DM}$, His-317 is not located over the heme center and its imidazole does not interact with the heme iron (Fig. 3, thin line). The mutation of Gly-310 ($P450_{14DM}$) to hydrophilic Asp in a rather hydrophobic environment would cause a shift of Asp-310 ($P450_{SG1}$) toward the protein surface where it would interact with water and/or basic amino acid residues such as Lys-176 and Arg-179 (not shown in Fig. 3). Translational and rotational shifts of Asp-310 along the helix would result in an effective interaction of His-317 residue with the heme iron (Fig. 3, thick line). Local deformation (not shown in Fig. 3) of the following region, Thr-318 to Ala-324, may be afforded by the presence of highly populous threonines and serines (24), and may facilitate the shift without significantly affecting

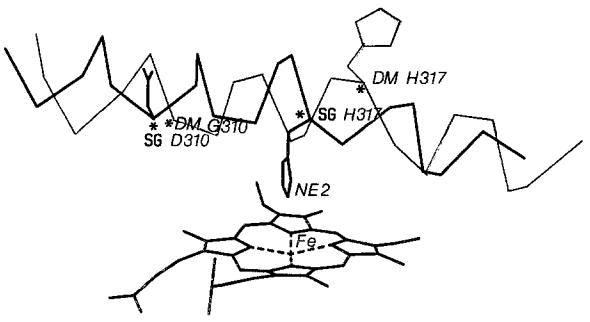

Fig.3 Structural model showing correlation between distal helices and heme molecule of $P450_{14DM}$ and $P450_{SG1}$. Residues 310 and 317 of $P450_{14DM}$ (DM) and $P450_{SG1}$ (SG) are labeled, * , on α-carbon backbone helices (thin line for $P450_{14DM}$ and thick line for $P450_{SG1}$) with their side chains. Shift of helix would result in the coordination of nitrogen atom (NE2) of histidine residue to the heme iron (Fe).

the conformation of the region that follows the helix (not shown in Fig. 3). Thus, we are fairly confident in the prediction that the 6th ligand to the heme iron of the inactive $P450_{SG1}$ is the imidazole group of His-317 residue. Further structural investigation of $P450_{14DM}$ and $P450_{SG1}$ using site directed mutagenesis and molecular modeling of the helix is in progress.

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