Purification of Cytochrome P-450*alk* from *n*-Alkane-grown Cells of *Candida maltosa*, and Cloning and Nucleotide Sequencing of the Encoding Gene

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When the cells of an *n*-alkane-assimilating yeast, *Candida maltosa* IAM12247, were transferred from a glucose medium to an *n*-alkane medium, various enzymes are induced in the endoplasmic reticulum and peroxisome. Cytochrome P-450*alk*, one of these enzymes in the endoplasmic reticulum, was purified after mild solubilization of the membrane, followed by a few steps of chromatography. The enzyme was characterized spectrophotometrically and its N-terminal amino acid sequence (12 residues) was determined.

Using oligonucleotide probes prepared to match parts of the N-terminal amino acid sequence and of the partial cDNA sequence of cytochrome P-450*alk* of *C. maltosa* EH15, we isolated from a gene library of *C. maltosa* IAM12247 a clone which had a gene encoding cytochrome P-450*alk*. By nucleotide sequencing of this gene, the amino acid sequence of this enzyme was deduced. It consisted of 523 amino acids (59,838 daltons), with a non-cleavable signal sequence in the N-terminal region. The structure of this enzyme was compared with some other members of the cytochrome P-450 superfamily.

Cytochrome P-450s (P-450s) are heme-containing monooxygenases which catalyze diverse oxidative reactions in the metabolisms of a large number of endogenous and xenobiotic compounds, and play the major roles in steroid biogenesis, drug metabolism, and chemical carcinogenesis. In higher eukaryotes, there is a P-450 superfamily and the multiple genes encoding P-450s support this diversity. Other than higher eukaryotes, the presence of P-450s is known in some bacteria and some lower eukaryotes such as yeasts. One of the best characterized P450s in yeasts is P-450*alk* in the *n*-alkane-assimilating *Candida* spp., in both biochemical^{1~10)} and molecular biological^{11,12)} aspects. This enzyme, coupled with NADPH-cytochrome P-450-reductase, catalyzes the terminal hydroxylation of *n*-alkane, the first and the rate-limiting step in the assimilation pathway.

Having a role in carbon source assimilation, the physiological significance of P-450*alk* is different from those of higher eukaryotes and another P-450 in yeasts, P-450*14DM*, which engages in the biogenesis of ergosterol, the yeast analog of cholesterol. P-450*14DM* has been found and analyzed in an *n*-alkane-assimilating *Candia* yeast,^{13~15)} *Candida albicans*^{16,17)} and *Saccharomyces cerevisiae*.^{13,18~21)}

The transfer of *n*-alkane-assimilating yeast cells from glucose- to *n*-alkane-medium results in the induction of various enzymes including P-450*alk*, and in the changes in the endoplasmic reticulum (ER) and peroxisome in which these enzymes are located.^{5~7)} It has been reported that inducibility of some of these proteins are regulated at the level of transcription.^{11,22,23)} To investigate the detailed mo-

Abbreviations: cytochrome P-450, P-450; ER, endoplasmic reticulum; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethylsulfoxide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; SSC, 0.15 M NaCl and 0.015 M sodium citrate; kb, kilobase pairs; ORF, open reading frame.

lecular mechanism of the regulation of gene expression and organelle biogenesis, we have developed genetic systems of *Candida maltosa*^{24~26)} and constructed host-vector systems.^{27~29)} In this paper are described the purification of P-450*alk* protein and the isolation and sequencing of a P-450*alk*-encoding gene from *C. maltosa*. The deduced amino acid sequence is compared to those of some other members of the P-450 superfamily to understand the structure-function relationships among P-450s.

Materials and Methods

Strains and media. The yeast strain used was *C. maltosa* IAM12247. The MY medium, the minimum medium (the MS medium) and growth conditions were as described previously.²³⁾ As a carbon source, 1% glucose or *n*-tetradecane was added to MS medium. The bacterial strains used were *Escherichia coli* MC1061 [*hsr hsm*⁺ *araD139* Δ (*ara-leu*)7697 *AlacX74* *strA* GalU⁻ GalK⁻] and MV1184 [*ara* Δ (*lac-pro*) *strA* *thi* (ϕ 80 *lacZ* Δ M15) Δ (*srl-recA*) 306::Tn 10 (*tet*⁺) F':*traD* 36 *proAB lacI*^{qZ} Δ M15], which was grown in Luria broth.

Purification of P-450*alk*. P-450*alk* was purified by a modified procedure of already published methods developed for purification from rat liver³⁰⁾ and from an *n*-alkane-assimilating yeast strain, *Lodderomyces elongisporus* EH "D",⁷⁾ which is now re-named as *Candida maltosa* EH15.^{9,10,22)} The cells of *C. maltosa* IAM12247 grown on *n*-tetradecane using a jar fermenter were collected at middle-log phase and stored at -80°C until use. Preparation of cellular extract, post-mitochondrial supernatant (the PM fraction), and microsomal fraction was done as described previously.²³⁾ A microsomal pellet obtained from 50 to 100 g of wet cells was suspended in 38 ml of the solubilizing buffer (50 mM Tris-HCl, pH 7.5, 20% (v/v) glycerol, 5 mM β -mercaptoethanol, 1 mM EDTA) containing 0.25 mM PMSF and 0.1% DMSO, and added to the same volume of 2.4% sodium cholate in the same solution to solubilize the microsomal membrane mildly. Then the solubilized microsomes were centrifuged at 105,000 $\times g$ for 120 min to obtain solubilized microsomal supernatant (the SM fraction). The SM fraction was put onto a 25-ml column of AH-Sepharose 4B (Pharmacia Co.), and P-450*alk*-rich fraction (the AH fraction) identified by spectrophotometrical analysis was eluted with about 70 ml of the solubilizing buffer containing PMSF and DMSO as described above and 0.4% sodium cholate and 0.55% Emulgen 913 (Kao Atlas Co.). About 140 ml of the AH fraction thus obtained were diluted with 420 ml of the solubilizing buffer to decrease the concentrations of

the detergents. The diluted AH fraction was then put onto a column of DEAE-Sepharcel (the volume was 14 ml, Pharmacia Co.) and eluted with a linear gradient of NaCl (from 30 mM to 120 mM) in the solubilizing buffer in which the concentration of Tris-HCl was decreased to 10 mM, containing 0.1% Emulgen 913. Each fraction (2.7 ml) was analyzed spectrophotometrically for P-450*alk* content, and those fractions rich in P-450*alk* were pooled (about 22 ml, the DE fraction). Ten ml of the DE fraction was put onto a column of Bio-Gel HTP (1 g, Bio-Rad Co.) and eluted with solutions of stepwise increasing concentrations (from 20 mM to 150 mM) of KH₂PO₄-KOH, pH 7.5. The P-450*alk*-rich fractions eluted with 80 mM and 100 mM of KH₂PO₄-KOH were pooled as the HA fraction. This HA fraction was used as purified P-450*alk* for characterization of this enzyme. To analyze N-terminal amino acid sequence, the DE fraction described above containing about 1 nmol of P-450*alk* was concentrated to about 100 μ l by a Minicon-B15 (Amicon Corp.) and put onto a column of TSK gel G3000SW (5.5 mm $\times$ 60 cm, Toyo Soda Co.) for HPLC. Elution was done with a solution containing 0.1% SDS, 4% glycerol, and 100 mM NaCl. A P-450*alk*-rich fraction of 300 μ l (the G fraction) was obtained and concentrated to about 100 μ l before analysis of the N-terminal amino acid sequence by automated Edman degradation in a model 470 protein sequencer (Applied Biosystems.)

Methods for characterization of P-450*alk*. SDS-PAGE was done as described by Laemmli³¹⁾ using 9% separating gels. Subunits of heat-resistant RNA polymerase B (Seikagaku Kogyo Co.) were used as molecular weight markers. Spectrophotometrical analyses of P-450*alk*³²⁾ and measurement of protein³³⁾ were done as described previously.

Construction of a gene library of *C. maltosa* genome. Total DNA of *C. maltosa* was isolated and genomic DNA library was constructed by a modified method described previously.²⁷⁾ In brief, after partial digestion with *Sau*3A1, 5~10 kb genomic DNA fragments were size-fractionated by sucrose density gradient centrifugation. The DNA fragments were ligated to *Bgl*II-digested and phosphatase-treated vector DNA of *C. maltosa*. This vector, pBTH10B, consisted of pBR322, the TRA sequence²⁷⁾ for autonomous replication in *C. maltosa*, and the *C-HIS5* gene (manuscript in preparation). Then, *E. coli* MC1061 was transformed with the ligated DNA.

Isolation of mRNA, synthesis of cDNA probes, and conditions of hybridization. Total RNA of *C. maltosa* grown on glucose or *n*-tetradecane was isolated by the method using guanidium thiocyanate-cesium chloride.³⁴⁾ Poly(A)⁺-RNA was selected by oligo(dT)-cellulose chromatography.³⁵⁾ A single strand cDNA probe labeled with [α -³²P]dCTP (Amersham Co.) was synthesized using a cDNA synthesis kit (Boehringer Mannheim Co.).

Differential hybridization was done as described previously.³⁶⁾ Colony-blotted or dot-blotted replica filters (Hybond-N, Amersham Co.) were hybridized with a cDNA probe in a solution containing $6\times$ SSC, $5\times$ Denhardt solution, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA, and 0.1 mg/ml denatured vector pBTH10B DNA after digestion with restriction enzymes. Hybridization was carried out at 65°C overnight.

Synthesis of oligonucleotide probes, conditions of hybridization, and DNA sequencing. Oligonucleotide probes were synthesized by the automated phosphoramidite method in a DNA synthesizer (Beckman Co.). They were labeled with [γ -³²P] ATP (Amersham Co.) by T4 polynucleotide kinase (Takara Shuzo Co.). Dot-blotted filters were hybridized with a probe at 58°C overnight in a solution containing $6\times$ SSC, $5\times$ Denhardt solution, 0.1% SDS, and 0.1 mg/ml denatured *E. coli* total DNA. Southern hybridization³⁷⁾ and colony hybridization³⁸⁾ were done as described previously. Restriction enzymes and the other enzymes used for DNA manipulation were purchased from Takara Shuzo Co. DNA sequencing was done by the dideoxy sequencing method³⁹⁾ using a kit, Sequenase (Toyobo Co.) or by a automated sequencer,⁴⁰⁾ Genesis 2000 DNA Analysis System of E. I. Du Pont. For computer analysis, Genetix software (SDC-software Development Co.) was used.

Results

Characterization of purified P-450alk

P-450alk was purified from the cells grown on *n*-tetradecane as a carbon source. The specific content and the yield of this enzyme in each step of the purification procedure are summarized in Table I. The final specific content was 9.3 nmol/mg protein. In SDS-PAGE, the HA fraction gave one clear major band of P-450alk and a few faint smear bands of contaminating proteins of high molecular weight. The apparent molecular weight by SDS-

PAGE was 56,000. The theoretical specific content of purified P-450alk is about 18 nmol/mg protein when it is calculated from the apparent molecular weight and an assumed millimolar extinction coefficient of 91³²⁾ for CO-difference spectra. According to this calculation, the purity of our P-450alk preparation was 52%, which was too low compared to the apparent purity judged from the pattern of SDS-PAGE. This difference may be due to the contamination of P-450alk with residual detergents which influence the Lowry method.⁷⁾

The oxidized form of the purified P-450alk showed a low spin type absorption spectrum with a Soret peak at 418 nm, an α peak at 568 nm, and a β peak at 534 nm. A Soret peak of the reduced form was at 420 nm. The reduced CO-complex gave a Soret peak at 450 nm.

The N-terminal amino acid sequence was analyzed using about 0.2 nmol of P-450alk of the G fraction; it was Ala-Ile-Glu-Gln-Ile-Ile-Glu-Glu-Val-Leu-Pro-Tyr-.

Molecular cloning and sequencing of a gene encoding P-450alk

A gene for P-450alk was cloned by a two-step procedure. First, since a gene for P-450alk is induced by *n*-alkane in the culture medium, we isolated from a gene library of *C. maltosa* genome those clones which were induced only by *n*-alkane but not by glucose, using a differential hybridization method. Two kinds of cDNA probes, one for mRNA from *n*-tetradecane-grown cells and the other for mRNA from glucose-grown cells, were hybridized separately with *E. coli* colonies carrying a *C.*

Table I. PURIFICATION OF CYTOCHROME P-450alk

Fraction	Protein (mg)	Cytochrome P-450 (nmol)	Specific content (nmol/mg protein)	Yield (%)
PM	1,100	39	0.034 (1) ^a	100
SM	130	26	0.19 (5.6)	68
AH	3.5	9.4	2.7 (80)	24
DE	0.95	4.2	4.4 (130)	11
HA	0.056	0.52	9.3 (270)	1.3

^a The ratio of the specific content of cytochrome P-450 in each fraction to that of the PM fraction.

Probe 1 (17mer)
 5'ATC-GAA-CAA-ATC-ATC-GA 3' (for Ile-Glu-Gln-Ile-Ile-Glu)
 T G G T T

Probe 2 (17mer)
 5'CAA-ATC-ATC-GAA-GAA-GT 3' (for Gln-Ile-Ile-Glu-Glu-Val)
 G T T G G

Probe 3 (28mer)
 5'GGTCCAAGAATTTGTTGGGTCAACAAT 3'

Probe 4 (21mer)
 5'AAACTTACCCACCAACAAAT 3'

Fig. 1. Synthetic Oligonucleotide Probes.

Probes 1 and 2 were synthesized to match the N-terminal amino acid sequence of purified P-450alk. All the possible codons were used, except for the ATA codon of Ile, which has been found to be a rare codon in the *C-LEU2* gene of *C. maltosa*.²⁵⁾ Probes 3 and 4 were synthesized to match the information on a partial nucleotide sequence of cDNA for P-450alk of *C. maltosa* EH15, which was kindly supplied by Dr. Schunck as described in the test.

Fig. 2. Restriction Map and Sequencing Strategy for the P-450alk gene.

The thin line indicates the vector sequence, and the open box indicates the *C. maltosa* DNA sequence. The big arrow represents the open reading frame in the sequenced region. Abbreviations: E, *EcoRI*; H, *HindIII*; S, *SalI*; P, *PstI*; T, *EcoT221*.

maltosa genome library. From about 60,000 clones about 2,000 were selected that hybridized specifically with cDNA for mRNA from *n*-tetradecane-grown cells. Then they were examined in the same way again, and about 300 positive clones were isolated. From each of these clones, plasmid DNA was isolated, and dot-blot-hybridization was done using two kinds of cDNA probes. About 50 positive clones were obtained, which were used in the second step.

Then the clones thus selected were screened to detect a clone carrying a gene for P-450alk, using four kinds of synthetic oligonucleotide

probes. Probes 1 and 2, which were mixtures of oligonucleotides, were synthesized to match parts of the N-terminal amino acid sequence of purified P-450alk (Fig. 1). Probes 3 and 4 were synthesized to match a partial nucleotide sequence of cDNA (kindly informed us by Dr. Schunck) for P-450alk of *n*-alkane-grown cells of *C. maltosa* EH15^{9,10,22)} (formerly named *Lodderomyces elongisporus* EH "D")⁷⁾ (Fig. 1).

The restriction map of the insert in the plasmid carried in this clone is shown in Fig. 2. After subcloning, an *EcoT221* DNA fragment having 2803 nucleotides was sequenced, which

starts with ATG at nucleotide 1,018 and ends with TAG at nucleotide 2587, comprising 1569 nucleotides and 523 codons. The deduced molecular weight of this ORF is 59,838, which is

Fig. 4. A Hydropathy Plot of P-450alk.

Hydrophobicity increases from $-$ to $+$. A standard algorithm with a window of 15 residues is used.

close to the apparent molecular weight (56,000) of purified P-450alk. The deduced sequence of the N-terminal 12 amino acids boxed in Fig. 3 is identical to that of the sequence of purified P-450alk shown above, except for the initial Met. There are sequences, underlined in Fig. 3, which are identical to those of probes 3 and 4, except for 1 at the nucleotide 2439.

Two presumptive TATA boxes and AATAAA boxes are found in the 5'- and 3'-region of the ORF, respectively, as indicated in Fig. 3.

Of 523 amino acids, 49.7% are hydrophobic residues (Gly, Ala, Val, Leu, Ile, Met, Phe, Trp, Pro). The hydropathy plot of P-450alk according to Kyte and Doolittle's algorithm⁴¹⁾ is shown in Fig. 4.

Comparison of the structure of P-450alk with those of other P-450s

Recently, Sanglard and Loper¹²⁾ analyzed the nucleotide sequence of DNA for P-450alk from *C. tropicalis* which is also inducible by and hydroxylates *n*-alkane. Figure 5 shows an alignment of the deduced amino acid sequences of P-450s from *C. tropicalis* (543 amino acids) and *C. maltosa* (523 amino acids). If some gaps are introduced, 524 are overlapped; 302 amino acids (57.6%) are

identical and 129 amino acids (24.6%) are conservatively replaced.

Alignment of our P-450alk with other kinds of P-450 in yeasts, P-45014DM from *S. cerevisiae*²⁰⁾ and from *C. tropicalis*¹⁵⁾, showed low homology. With P-45014DM from *S. cerevisiae* (530 amino acids), 444 are overlapped and 77 (17.3%) are identical. With that from *C. tropicalis* (528 amino acids), 373 are overlapped and 68 (18.2%) are identical. Homologous regions are limited only to the C-terminal part, including the HR2 region (see below). This indicates that functionally different P-450s in yeasts, P-450alk and P-45014DM, are also different in their structures.

Comparison to the other 8 typical P-450s from 7 vertebrates^{42~48)} and from *Pseudomonas putida*⁴⁹⁾ was done by both sequence alignment and dot matrix. Although homology is low, there are some conserved regions. One of such regions is the previously designated HR2 region.^{50,51)} This region, boxed in Fig. 5, contains an invariant cysteine, which is thought to be the fifth ligand to heme.⁴⁹⁾ Other conserved regions, underlined in Fig. 5, are in the C-terminal part of the molecule; a heme-distal helix containing threonine,⁴⁹⁾ and a sequence with a tridecapeptide.⁵²⁾

Among 7 vertebrate P-450s compared, P-450pcnl and P-450L ω have high similarity to

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1'      MAIEQIIIEEVLPLYLTKWYTLFGAAVTYFLSIALRNKFYEYKLCEN-
      *...*      **** .. .. *      *...*
1" MSSSPSIAQEFLATITPYVEYQCENYTKWYFIPLVILSLNLISMLHTKYLERKFKAKPL

48' P VYFEDAGLFGIPALIDI I KVRKAGQLADYDTDTTFDKYPNLS--SYMTVA-GVLKI--VF
      ** .. *...* .. * .. * .. * .. * .. * .. * .. * .. * .. * .. *
61" AVYVQDYTFCLITPLV-LIYYKSKGTVMQFACDLWDKKLIVSDPKAKTIGLKILGIPLIE

103' TVDPENIKAVLATQFNDFALGARHAHFDPLLGDGIFTLDGEGWKL SRAMLRPQFAREQIA
      * ****,* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
120" TKDPENVKAILATQFNDFSLGTRHDFLYSLLGDGIFTLDGAGWKIISRTMLRPQFAREQVS

163' HVKALEPHVQILAKQIKLNKGKTFDLQELFFRFTVDTATEFLFGESVHSLYDEKLG I-PA
      *** ****,* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
180" HVKLLLEPHMQVLFKHIRKHHGQTFDIQELFFRLTVDSATEFLGESAESLRDES VGLTPT

222' PNDIPGRENFAEAFNTSQHYLATRTYSQIFYWLTNPKEFRDCNAKVHKL AQYFVNTALNA
      ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
240" TKDFDGRNEFADAFNYSQTNQAYRFLQMYWILNGSEFRKSIAIVHKFADHIVQKALEL

282' TEKEVEEKS KGGYVFLYELVKQTRDPKVLQDQLLNIMVAGRDTTAGLLSFAMFELARNPK
      *... ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
300" TDEDL-EK-KEGYVFLFELAKQTRDPKVL RDQLLNILVAGRDTTAGLLSFLFFELSRNPE

342' IWNKLEEEVFNFLGDEARVDEISFETLKKCEYLKAVLNETLRMPSPVPINFRTATRDT
      * ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
358" IFAKLREEIENKFGLGQDARVEEISFETLKSCEYLKAVINETLRIYPSVPHINFRVATRNT

402' TLPRGGGKDGNSPIFVPKGSSVVYSVYKTHRLKQFYGEDAYEFRPERWFEPSTRKL GWAY
      ***** ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
418" TLPRGGGEGGLSPIAIKKGQVVMYTLATHRDKDIYGEDAYVFRPERWFEPETRKL GWAY

462' LPFNGGPRICLGQQFALTEASYV IARLAQMFEHL-ESKDETYPPNKC IHLTMNHNEGVFI
      ***** ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..* ..*
478" VPFNGGPRICLGQQFALTEASYVTVRLQEFGNLQDPNTEYPPKLQNTLTLSLFE GA EV

521' SAK

538" QMYLIL

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Fig. 5. Comparison of the Amino Acid Sequence of P-450_{alk} of *C. maltosa* (the upper sequence, 523 residues) with That of *C. tropicalis*¹²⁾ (the lower sequence, 543 residues).

Asterisks indicate identical amino acids, and dots indicate conservatively replaced amino acids in both sequences. Dashes indicate gaps introduced to obtain maximal homology. The HR2 region which contains an invariant cysteine is boxed. Three hydrophobic segments in the N-terminal region are overlined. Other two conservative regions are underlined.

P-450_{alk}; alignments are possible in almost all regions and identical amino acids are 25.6% and 24.1%, respectively. This suggests that these P-450s may be closer to P-450_{alk} functionally or evolutionarily. In fact, the substrate of P-450_{LAω}, lauric acid, resembles the structure of *n*-alkane, the substrate of P-450_{alk}.

Discussion

Cytochrome P-450 from *n*-alkane assimilating yeast was first purified and characterized by Riege *et al.*⁷⁾ from *C. maltosa* EH15. The specific content of their purified P-450_{alk} was about 11 nmol/mg protein. We have tried to trace their experiment to purify P-450 from *C. maltosa* IAM12247, but the specific content and the yield were not as good as expected.

This difference might be due to the difference of the strains used or of the techniques used during purification in Riege's group and our group. So we modified the procedure of purification of P-450alk and finally succeeded in purification of P-450alk to almost the same extent as reported by Riege *et al.*⁷⁾

Although the purity of our P-450alk was theoretically calculated to be 52%, we concluded that the real purity of our preparation judged from the pattern of SDS-PAGE was high enough to be analyzed for its properties and its N-terminal amino acid sequence. In fact, we could determine 12 amino acid residues at the N-terminus. And with information of the N-terminal amino acid sequence and the internal nucleotide sequence of cDNA of P-450alk of *C. maltosa* EH15 supplied by Dr. Schunck, we could isolate and sequence a gene encoding P-450alk from *C. maltosa* IAM12247.

The conclusion that the ORF found in the isolated and sequenced DNA fragment shown in Fig. 3 and Fig. 4 encoded the structural gene for P-450alk, was drawn from the following reasons. 1) The expression of this gene is inducible by *n*-alkane and not by glucose. 2) The N-terminal amino acid sequence of the coding ORF is identical to that of purified P-450alk, except for the initial Met. 3) The deduced molecular weight of the ORF is very close to the molecular weight of purified P-450alk. 4) It has sequences almost identical to those of the probes, which were prepared according to the sequence data of cDNA for P-450alk from *C. maltosa* EH 15. 5) Its sequence has high homology to that of DNA for P-450alk of *C. tropicalis*. The last item might also indicate that the genomic DNA sequence of P-450alk of *C. maltosa* does not contain any intron, since it was suggested previously that the genomic sequence of P-450alk of *C. tropicalis* does not have any intron.¹²⁾ This suggestion is supported by the fact that in the ORF there is no TACTAAC sequence which is found typically in the middle of introns of the genes from *S. cerevisiae*.⁵³⁾

Most of the members of the P-450 super-

family so far analyzed are membrane-associated proteins in the ER, and so is the case of P-450alk. The extremely hydrophobic N-terminal region, which is commonly found in these P-450s in the ER, is believed to be responsible for both a signal-recognition-particle dependent, non-cleavable insertion signal into the ER membrane and a stop-transfer sequence.^{54,55)} The N-terminal amino acid sequence of purified P-450alk and the nucleotide sequence of the ORF in the cloned DNA fragment (Fig. 2) indicate that P-450alk is not cleaved like the other P-450s in the ER. It is found that the N-terminal region has the highest hydrophobicity in P-450alk (Fig. 3), which is composed of three hydrophobic segments overlined in Fig. 5; the first 25 amino acid segment from position 10 to 34, the second 13 amino acid segment from 54 to 66, and the third 12 amino acid segment from 92 to 103. Probably the first segment is mainly responsible for translocation of this protein into the membrane on the basis that it is extremely hydrophobic, that it has a proper length required for membrane spanning, and that it is followed by a sequence with charged amino acid residues proposed as a putative halt-transfer signal. The second and third segments are followed by charged residues too, but they are slightly too short to span the membrane. Between these three segments, there are considerably hydrophilic segments. This typical feature leads us to think that the N-terminal part of P-450alk may cross the ER membrane a few times.

One of our main interests is to understand the molecular mechanisms of induction of various genes of enzymes by *n*-alkane in *C. maltosa*, which we believe is a suitable model system to investigate regulation of gene expression by hydrophobic compounds. The cloning of these genomic genes is a necessary step for this purpose. The next step would be to assay promoter activity of the P-450alk gene in the presence of various carbon sources, to analyze *cis*-element(s) in the 5'-flanking region of the gene responsible for induction, and to find *trans*-element(s) which interacts with the

cis-element(s). For these kinds of investigations, out host-vector system of *C. maltosa*^{27~29)} should be powerful and indispensable tools. Furthermore, other genes for proteins induced by carbon sources in *n*-alkane-assimilating yeasts such as peroxisomal fatty acyl coenzyme A oxidase^{56,57)} will also be useful for these investigations. Isolation of genes with this characteristic other than the gene for P-450*alk* is being done in our laboratory using the clones isolated by the differential hybridization experiments described in this paper.

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