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Relation Between Evolutionary Distance and Enzymatic Properties among the Members of the CYP52A Subfamily of *Candida maltosa*

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The CYP52A subfamily of the alkane-assimilating yeast *Candida maltosa* consists of six structurally related isoforms. Four of them (CYP52A3, 4, 5, and 9) are strongly induced by alkanes and play an important role for the conversion of various alkanes and fatty acids. Taking advantage of a homologous overexpression system, we found in the present study that both of the two other CYP52A forms, CYP52A10 and CYP52A11, represent specialists for the hydroxylation of lauric acid suggesting their preference for short-chain fatty acids. At the same time, they hydroxylated palmitic acid only moderately and failed to convert hexadecane. Based on the now completed knowledge about the principal substrate specificities of all members of the CYP52A subfamily of *C. maltosa*, it became apparent that evolutionarily more distantly related P450 forms developed either to alkane or to fatty acid hydroxylases, whereas P450 forms which retained the ability to convert both types of substrates were also found to be evolutionarily related to both alkane and fatty acid hydroxylases.

Cytochromes P450 (P450s) constitute a superfamily of ubiquitous haem-thiolate proteins (1) which catalyze oxidations of a wide variety of endogenous and xenobiotic compounds (2). Higher eukaryotes generally contain several different P450 families which often effect cellular processes as an ensemble of their isoforms. In microorganisms, however, an extended P450 multiplicity which approaches that of some mammalian P450 families has been detected only in some *Candida* species (3-6). These P450s constitute the CYP52 family and catalyze the terminal hydroxylation of n-alkanes which represents the first and rate-limiting step in the alkane degradation pathway, and the ω-hydroxylation of fatty acids, thus providing the molecular basis for the alkane-utilizing phenotype of those yeast species (7). In *Candida maltosa*, the CYP52 family consists of eight structurally related isoforms, not counting the allelic variants present: six P450s belonging to the CYP52A subfamily, CYP52C2 and CYP52D1 (5). As shown previously, four P450s of the CYP52A subfamily are strongly induced by n-alkanes (CYP52A3, 52A4, 52A5 and 52A9), whereas the other two (CYP52A10 and 52A11) as well as CYP52C2 and 52D1 are only poorly or not inducible by these compounds (5). Taking advantage of heterologous expression in *Saccharomyces cerevisiae*, the four major alkane-inducible P450 forms of *C. maltosa* were enzymatically characterized (8-10). Testing various alkanes and fatty acids, distinct preferences of individual P450 forms concerning substrate class and chain length were detected (9).

In order to complete our knowledge about the physiological significance of the whole CYP52A subfamily of *C. maltosa*, the present study was aimed at getting insight into the individual substrate specificities of the other two CYP52A forms, CYP52A10 and CYP52A11. Moreover, the information about enzymatic properties of all members of the CYP52A subfamily now enables us to provide interesting insight into P450s structure-function relationship in an evolutionary context.

MATERIALS AND METHODS

Plasmids, strains, and culture conditions. Construction of plasmids pNGH2-Alk1, pNGH2-Alk7 and pNGH2-Alk8 for overexpression of CYP52A3, 52A10 and 52A11, respectively, was described previously (11). *C. maltosa* strain CHA1 (*his5*, *ade1;* 12) which was used as a host for P450 overproduction was transformed by a lithium acetate method (13), and cultivated at 30 °C in 5 l shaking flasks containing 1.5 l yeast minimal medium (1.34% yeast nitrogen base without amino acids, Difco), 100 mg adenine/l, 0.5 mg $FeCl_3$/l and 2%

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glucose as the carbon source. To induce P450 transcription from the *GAL1* promoter, the cultures were harvested at a cell density of 1 to 2 x 10^8 cells/ml, the cell pellet was washed with glucose-free medium and resuspended for further cultivation in fresh medium containing 2% galactose. Maximal P450 levels were reached after an induction time of 14 h. P450 was quantified by means of CO difference spectra using an extinction co-efficient of 91 mM^{-1} x cm^{-1} (14).

Preparation of microsomes. Cells were harvested and disrupted mechanically in 50 mM Tris/HCl, pH 7.4, 400 mM sorbitol, 0.5 mM dithiothreitol, 1 mM EDTA and 15 % glycerol by means of glass beads (0.45–0.5 mm) using a small scale cell homogenizer (Braun Melsungen AG, Germany). After two centrifugation steps (3,000 x g for 5 min and 10,000 x g for 10 min), microsomes were obtained from the remaining supernatant by $CaCl_2$-mediated membrane aggregation as previously described (10).

Cytochrome P450 activity assay. For the determination of microsomal P450 activities, the enzyme assay used corresponded to the method described in (10). As substrates, the following radiolabelled compounds were used: [^{14}C]hexadecane (Amersham; 9.5 MBq/mmol), [^{14}C]lauric acid (Amersham; 27.0 MBq/mmol), [^{14}C]palmitic acid (Amersham; 23.6 MBq/mmol). The final substrate concentrations were adjusted to 1.0 mM (alkane) and 0.5 mM (fatty acids). Emulgen 913 was added to the hexadecane hydroxylation assays (0.02%) to improve substrate transfer. Product separation was performed by TLC followed by quantification of the [^{14}C]-labelled metabolites using the BAS2000 Bioimagine analyzer (Fuji Film).

Multiple P450 sequence alignment. The automated multiple sequence alignment and the phylogenetic tree of all six CYP52A forms were created using the CLUSTAL V program (provided via the world wide web by the European Bioinformatics Institute; 15).

RESULTS AND DISCUSSION

Substrate specificity of CYP52A10 and CYP52A11. To characterize both *C. maltosa* CYP52A forms which are poorly or not inducible in the presence of alkanes (5), we took advantage of the *C. maltosa* overexpression system previously established in our lab (11, 16 and references therein). It has been demonstrated previously that *C. maltosa* shows a deviation from the universal genetic code by translating the codon CUG as serine instead of leucine (17, 18). Therefore, compared to the *Saccharomyces cerevisiae* host/vector system which was applied to determine the enzymatic properties of the other CYP52A forms (9, 10), the homologous *C. maltosa* expression system did not require a replacement of all the CTG triplets by a serine codon to obtain the wild-type CYP52A10 and 52A11 proteins.

As the first step, we placed the coding regions of CYP52A10 and CYP52A11 under control of the *GAL1* promoter in plasmid pNGH2, as previously reported (11, 16). After transformation of *C. maltosa* CHA1 (12), respective cultures were grown on glucose to a cell density of 1 to 2 x 10^8 cells/ml and then shifted to galactose-containing medium to induce P450 transcription. After P450 accumulation to about 40 nmol/l culture medium, microsomal fractions were isolated and tested for hydroxylation activities toward alkane (hexadecane) and fatty acids (lauric acid, palmitic acid). For a comparison with the data previously obtained after heterologous expression in *S. cerevisiae* (9), also CYP52A3 containing microsomes were investigated which were isolated from the respective *C. maltosa* overexpression strain.

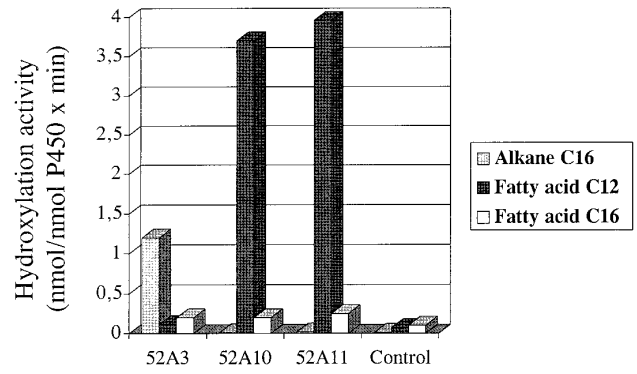

FIG. 1. Hydroxylation activities of CYP52A3, 52A10 and 52A11. Hexadecane (Alkane C16), lauric acid (Fatty acid C12) and palmitic acid (Fatty acid C16) hydroxylation activities were calculated from 3 independent assays with standard deviations less than 10 %. Control microsomes were isolated from *C. maltosa* CHA1 transformed with plasmid pNGH2 (11). The ratios of hexadecane to lauric acid hydroxylation activities of CYP52A3 were found to be rather independent from the host/vector system used to overexpress the P450 form (ratios of 8.1 and 9.6 for microsomal fractions isolated from induced *C. maltosa* and *S. cerevisiae* cultures, respectively; compare reference 9).

As expected, these control microsomes containing CYP52A3 exhibited significant hexadecane hydroxylation activities, but clearly lower fatty acid turnover rates (Fig. 1). In contrast, CYP52A10 and 52A11 could not convert hexadecane, and hydroxylated palmitic acid only moderately. However, lauric acid was found to be a very efficient substrate for both P450 forms (Fig. 1). Thus, CYP52A10 and 52A11 which are 93 % identical in their primary structures could not be distinguished by their substrate specificities. Both hydroxylated lauric acid efficiently indicating their preference for short-chain fatty acids. The lack of hexadecane hydroxylation activity correlates not only with previous Northern blotting data which demonstrated a poor induction of CYP52A10/52A11 by alkanes (5), but it supports, in particular, our recent P450 gene disruption experiments (7). In this study, we found that the *C. maltosa* disruptant strain DA1235 which is defective in the expression of the major alkane inducible P450s CYP52A3, 52A4, 52A5 and 52A9, but still contained intact loci for CYP52A10 and 52A11, failed to grow on alkanes. Taken together with the *in vitro* characteristics of these enzymes as revealed in the present study, it can be clearly concluded that CYP52A10 and 52A11 are not able to initiate the alkane degradation pathway of *C. maltosa*. In the metabolic context, however, their ability to catalyze the hydroxylation of short-chain fatty acids originating from the action of the other P450 forms may be important to produce dicarboxylic acids for subsequent degradation by the peroxisomal β-oxidation system.

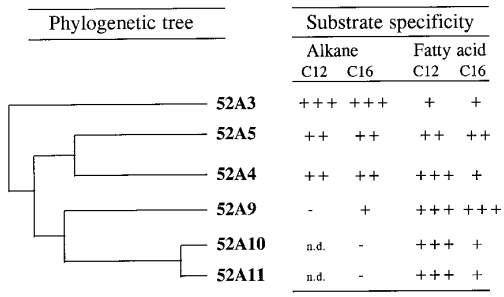

FIG. 2. Relation between evolutionary distance and substrate specificity among the members of the CYP52A subfamily of *C. maltosa*. The phylogenetic tree was established on the basis of a multiple sequence alignment done with the CLUSTAL V program (see Materials and Methods). Despite the application of various combinations of reasonable input parameters, the phylogenetic tree did not differ significantly which is likely due to the relatively high degree of sequence homology (56 to 93 % identity; 5). Interestingly, P450s which are tandemly encoded on the chromosome (CYP52A4 and 52A5 as well as CYP52A10 and 52A11) show not only a relatively high degree of sequence homology, but also common enzymatic and induction properties (see reference 5 for P450 induction data), suggesting that gene duplications were important events to create this functionally diverse P450 subfamily. For accession numbers and sequence homology data see (5). Turnover rates of CYP52A3, 52A4, 52A5 and 52A9 are presented in (9). Alkane C12–dodecane; Alkane C16–hexadecane; Fatty acid C12–lauric acid; Fatty acid C16–palmitic acid; n.d.–not determined.

Relation between evolutionary distance and substrate specificity among the CYP52A subfamily of C. maltosa. Including the present data on CYP52A10 and 52A11, all members of the *C. maltosa* CYP52A subfamily are now characterized with respect to their ability to convert alkanes and fatty acids of different chain length. This knowledge should enable us to get more insight into P450s structure-function relationship in an evolutionary context. To address the question of whether such a relation between the evolutionary distance and the enzymatic properties may be found, we created a phylogenetic tree of these P450s and compared their evolutionary position with their substrate specificities. Indeed, as shown in Fig. 2, such a structure-function relationship obviously exists. CYP52A3 which represents the most abundant and active alkane hydroxylase of *C. maltosa* (3, 4, 9) preferentially catalyzes the terminal hydroxylation of alkanes of different chain length, but is much less active toward fatty acids. On the other hand, CYP52A10 and 52A11, which are most distantly related to CYP52A3, were not able to convert hexadecane, but exhibited a remarkable lauric acid hydroxylation activity (compare Fig. 1). Thus from an evolutionary point of view, the alkane hydroxylase CYP52A3 is most distant from the short-chain fatty acid hydroxylases. Furthermore, CYP52A5 and 52A4, which are evolutionarily between CYP52A11 and 52A3, exhibited a less pronounced preference for one of the two substrate classes. CYP52A5 was even found to hydroxylate both alkanes and fatty acids of different chain length with a similar efficiency (9). Finally, CYP52A9 which is more closely related to CYP52A10 and 52A11 shares the lauric acid hydroxylation properties with those P450s, but its structure developed during evolution to convert also other long-chain fatty acids. At the same time, it retained structural elements for a less pronounced alkane hydroxylation activity which is still sufficient for *C. maltosa* to grow on hexadecane, but not on dodecane (7).

In conclusion, we assume that the CYP52A subfamily whose members are all located on the same *C. maltosa* chromosome (5) developed from an ancestral gene probably by gene duplication events and subsequent mutations within the P450s structure including the 6 assumed substrate recognition sites (SRS; 19, 20). As a result, an ensemble of P450s was created which allows the yeast *C. maltosa* to efficiently utilize n-alkanes and fatty acids over a broad chain-length range via mono- and diterminal oxidation reactions. The evolutionary process occurred under two aspects. First, P450s were adapted to the utilization of a certain chain length of the substrates and, second, to the conversion of a certain substrate class which required obviously more dramatic structural changes (see CYP52A3 and 52A11 in Fig. 2). As a result of this specialization process, the efficiency of substrate conversion could be increased (e.g., compare alkane conversion abilities of CYP52A3 and 52A5, and fatty acid conversion ability of CYP52A9 and 52A5), which likely provides a selection advantage for *C. maltosa* to utilize long-chain hydrocarbons in the environment.

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