Generation of the Soluble and Functional Cytosolic Domain of Microsomal Cytochrome P450 52A3*

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The cytosolic domain of microsomal P450 52A3 (P450Cm1) was isolated as a soluble and functionally active protein. The NH2-terminal region that anchors the P450 protein to the endoplasmic reticulum was removed by sequence-specific proteolysis at a designed cleavage site. For that purpose, P450Cm1 was genetically engineered to establish at position 63–66 the sequence Ile-Glu-Gly-Arg, which is recognized by the restriction protease factor Xa. The modified P450 was produced in high yields as an integral membrane protein in Saccharomyces cerevisiae. In the microsomal fraction, it was accessible to factor Xa digestion, releasing a readily soluble, shortened P450 protein. For large scale preparation of the cytosolic domain, the modified P450Cm1 was first purified and then subjected to sequence-specific proteolysis. The highly purified Δ(1–66)P450Cm1 exhibited unchanged spectral characteristics and catalyzed the hydroxylation of n-hexadecane with 85% of the activity determined for full-length wild-type P450Cm1. The method developed for the preparation of the cytosolic domain of P450Cm1 may be more generally applicable to facilitate structure-function studies on membrane-bound P450 forms.

Cytochromes P450 constitute a superfamily of ubiquitous heme-cholate proteins that catalyze diverse monoxygenase reactions in the metabolism of a wide variety of endogenous and xenobiotic compounds (1). As summarized recently (2), more than 200 individual P450 genes have been identified. Despite this large and steadily increasing number of primary structures available, to date crystallization and resolution of atomic structures has succeeded only in two soluble bacterial forms: P450cam (3) and P450BM3 (4). Their structures serve as a model to understand structure-function relationships of eukaryotic P450 forms, which in general, however, are only distantly related in terms of their amino acid sequences. Moreover, most of them are integral membrane proteins of the endoplasmic reticulum and a detailed investigation of their structures has been hindered by experimental problems in obtaining suitable crystals for x-ray analysis.

As known from studies with some other membrane proteins, the difficulties in crystallizing the proteins could be overcome after removal of their hydrophobic membrane anchor sequences (5). This strategy also applies promising in the case of microsomal P450 proteins on the condition that they are anchored to the membrane by only one or two NH2-terminal hydrophobic segments, whereas their catalytic domains should be largely exposed to the cytoplasm. Despite the fact that this model of membrane topology has been widely accepted (5, 7), it is still under discussion (8) and requires direct experimental evidence.

This question has been addressed in the present studies, which were aimed at the purification and functional characterization of the cytosolic domain of P450 52A3 (P450Cm1).1 P450Cm1 represents the major alkane-inducible P450 form of the yeast Candida maltosa (9–12). Expression of its cDNA in Saccharomyces cerevisiae resulted in the formation of an intact microsomal P450 catalyzing the terminal hydroxylation of long chain n-alkanes and fatty acids (13). High level expression of functionally active P450Cm1 seems to require membrane integration and to be supported by a massive proliferation of the endoplasmic reticulum, which bears the protein produced (13). As shown previously (14), deletions in the cDNA region encoding the putative membrane-anchor sequence of P450Cm1 led to the formation of truncated proteins, which were predominantly localized in the cytoplasm. However, this approach was not applicable for production of large amounts of the desired cytosolic domain of P450Cm1, probably because of its instability and rapid degradation in S. cerevisiae. Therefore, we searched for a strategy that allows maintenance of high level expression of P450Cm1 still in the native membrane-bound state and preparation of its cytosolic domain in a subsequent step. As shown here, this aim can be achieved by means of sequence-specific proteolysis.

EXPERIMENTAL PROCEDURES

Recombinant DNA Manipulations, Strains, and Culture Conditions—Recombinant PCR (15) was applied to alter three codons in the P450Cm1 cDNA (9) as shown in Fig. 1. The following oligonucleotides were used as primers: outside primer 1 (5’-AAATGCATGGCCATGCTAATAAAC-3’, annealing to position 15–33 of the P450Cm1 cDNA and containing a 5’-added SalI site), inside primer 1 (5’-ACTTCTTCTACCTTCGATTAAGCTGGGATCA-3’, annealing to position 191–223 of the P450Cm1 cDNA and containing the desired sequence alterations), inside primer 2 (5’-TTAATCGAAGGTAGAAAATAGAAAAGAAMGCAGGT-3’, annealing to position 204–236 of the P450Cm1 cDNA and overlapping with inside primer 1), and outside primer 2 (5’-CCCGATCTCTAGATGCTTTTATTTTTTTTTAA-3’, annealing to the 3’-end of the cloned P450Cm1 cDNA and containing the flanking restriction sites BamH1 and XhoI). In a first step, two separate reactions were performed using outside primer 1/inside primer 1 and inside primer 2/outside primer 2, respectively. The partially overlapping DNA fragments obtained were purified, mixed, and recombined in a subsequent PCR step using outside primer 1/outside primer 2. The resulting (Xa)P450Cm1 cDNA was ligated into the SalI/BamHI site of the expression plasmid Yep51 and

1 The abbreviations used are: P450Cm1 or cytochrome P450 52A3, the trivial name for the product of the CYF'52A3 gene (2); CHAPS, N,N,N-trimethyl-N-methylpropane-1,2-diol; DTTP, 1,2-dilauroylglycerol-3-phosphocholine; DTT, dithiothreitol; PCR, polymerase chain reaction; (Xa)P450Cm1, P450Cm1 containing a recognition site for factor Xa; FPLC, fast protein liquid chromatography.
transformed into \textit{S. cerevisiae} GRF 18 (MAT\textit{a}, his 3-11, his 3-15, leu 2-3, leu 2-112, can\textit{a}). The plasmid and the yeast strain were kindly provided by J. R. Broach (16) and D. Sanglard (17). Yeast transformants were cultivated in a 20-liter bioreactor on yeast minimal medium containing 1.94% yeast nitrogen base without amino acids (prepared according to Difco with slight modifications), 100 mg/liter \textit{L}-histidine and 2% glucose as initial carbon source. Cultivation was performed at 28°C, pH 4.8, and a dissolved oxygen tension of at least 70% saturation. Heterologous P450 expression was induced after complete glucose consumption by addition of galactose to a final concentration of 3% (w/v).

After 22 h of induction, cells were harvested by centrifugation.

**Purification of Recombinant P450 Proteins**—The harvested cells were disrupted mechanically as described (18). Microsomes were obtained from the 10,000 x g supernatant by CalCl\textit{m}-mediated aggregation. The microsomal P450 forms were solubilized with sodium cholate and purified on \omega-amino-\textit{n}octyl-Sepharose 4B as described (18). The pooled P450 fractions were loaded onto a hydroxyapatite column, equilibrated with 10 mM buffer A (potassium phosphate buffer, pH 7.3, 20% glycerol, 0.5 mM DTT, and 1 mM EDTA) containing 0.3% sodium cholate. The column was washed with 10 volumes of equilibration buffer, and the bound P450 protein was eluted with a linear 0.15-0.5 M sodium phosphate gradient.

**Sequence-specific Proteolysis**—For buffer exchange, the highly concentrated (Xa)P450Cml obtained (4 mg/ml) was passed through a Superose 12 gel filtration column (Pharmacia LKB Biotechnology Inc.), equilibrated with 0.1% (w/v) CHAPS in cleavage buffer (50 mM Tris/Cl, pH 7.8, 100 mM NaCl, 1 mM CaCl$_2$, 1 mM EDTA, 0.5 mM DTT, 20% glycerol). Site-specific cleavage was performed in the same buffer system containing 2% CHAPS. Factor Xa (Boehringer Mannheim) was added as a 1 mg/ml solution to give a P450:factor Xa ratio of 10:1. After a typical cleavage experiment 200 nmol (12 mg) of (Xa)P450Cml were treated in a total volume of 10 ml with 1.2 mg of factor Xa for 6 h at 4°C. If required, the sample was stored in liquid nitrogen until further use.

**Purification of Shortened P450Cml**—After proteolysis, the sample was loaded onto an \textit{n}-octyl-Sepharose CL-4B (Pharmacia) column (170 ml gel volume), equilibrated with 500 mM buffer A containing 0.1% sodium cholate and 0.5 mM phenylmethanesulfonyl fluoride. The column was then washed with three volumes of equilibration buffer, thereby eluting the shortened P450Cml as a broad red band. Residual uncleaved (Xa)P450Cml was eluted only after addition of Emulgen 911 (0.5% final concentration). Fractions containing the shortened P450Cml were pooled and dialyzed against 10 mM buffer A for final detergent removal, a hydroxyapatite step was performed as described above, with the exception, that detergents were omitted from all buffers.

The purified shortened protein was free of factor Xa contaminations as measured with the synthetic substrate \textit{n}-benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide (19).

**Molecular Weight Determination**—The molecular weight of the purified wild-type P450Cml and the shortened P450Cml were determined after detergent removal on hydroxylapatite by gel filtration using a Superdex 200 FPLC column (Pharmacia). FPLC was run in a buffer containing 10 mM potassium phosphate, pH 7.3, 0.15 M NaCl, 1 mM EDTA, and 0.5 mM DTT at a flow rate of 0.5 ml/min at 4°C. Blue dextran (M$_r$ = 2,000,000), ferritin (M$_r$ = 440,000), aldolase (M$_r$ = 180,000), bovine serum albumin (M$_r$ = 67,000), ovalbumin (M$_r$ = 43,000), and chymotrypsinogen A (M$_r$ = 25,000) were used as standards to calibrate the column. The calibration curve was obtained by plotting their $K_v$ values (elution volume of the protein - void volume)/(total bed volume - void volume) versus the logarithm of their molecular weight.

**Studies on Membrane Insertion**—To test the membrane integration of (Xa)P450Cml, microsomes were suspended in 0.1 M sodium carbonate, pH 11.5, to give a solution of 2 mg of protein/ml. After a 30-min incubation at 4°C, the suspension was centrifuged at 200,000 x g for 1 h. The supernatant was then precipitated with 10% trichloroacetic acid, rinsed with acetone, and dissolved in Laemmli sample buffer (20). The pellet was resuspended in the same volume of Laemmli sample buffer.

To study the role of the NH$_2$-terminal region in membrane integration, microsomes containing (Xa)P450Cml were suspended in cleavage buffer (3 mg of protein/ml) without addition of CHAPS and treated with factor Xa at a protease:protein ratio (w/w) of 1:50 for 12 h at 4°C. The cleavage mixture was then centrifuged for 1 h at 200,000 x g. Pellet and supernatant fractions were prepared for polyacrylamide gel electrophoresis as described above.

**Activity Measurements**—The following components were mixed in 10 mM buffer A containing 5 μM FAD/FMN and 0.5% sodium cholate: 0.5 nmol of P450, 0.05-5 nmol of NADPH/cytochrome P450 reductase, purified from \textit{C. maltosa} (21), and 700 μg of DLPC (sulfonated with sodium cholate at a detergent/phospholipid ratio of 2:1). Reconstitution was achieved removing sodium cholate by dialysis against 10 mM buffer A containing 5 μM FAD/FMN. Catalytic activities were determined by measuring the hydroxylation of n-[1-14C]hexadecane as described previously (13).

**Other Methods**—Established methods were used for the determination of P450 (22) and protein concentrations (23) and for SDS-polyacrylamide gel electrophoresis (20). Western blot analysis was performed as described previously using antibodies against P450Cml (13).

**RESULTS**

**Insertion of a Recognition Site for Sequence-specific Proteolysis**—As shown in Fig. 1, the P450Cml CDNA was genetically engineered to encode a modified P450Cml protein, designated as (Xa)P450Cml. It was identical to the wild-type protein, except that 3 amino acids were exchanged to establish the sequence Ile-Glu-Gly-Arg at position 63-66. This tetrapeptide should serve as recognition site for blood coagulation factor Xa, a restriction protease known to cleave immediately after this sequence (24).

**High Level Expression in S. cerevisiae**—The cDNAs encoding wild-type P450Cml and (Xa)P450Cml, respectively, were expressed in \textit{S. cerevisiae} under control of the galactose-inducible GAL10 promoter. For large scale production, the corresponding yeast transformants were cultivated in a 20-liter bioreactor, resulting in similar expression levels for both the P450 forms: 0.18 nmol of P450Cml and 0.12 nmol of (Xa)P450Cml/10$^8$ cells were determined by means of CO difference spectra of intact

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**Fig. 1.** Schematic representation of the NH$_2$-terminal amino acid sequence of P450Cml and its modification to create a recognition site for the restriction protease factor Xa. Shaded boxes denote the hydrophobic segments HS1 and HS2 included in the putative membrane-anchor region. The detailed part shows the required sequence alterations. The 4 amino acids forming the protease recognition site in the modified protein (designated as (Xa)P450Cml) are marked by asterisks.
yielding the cytosolic domain of P450Cml (lower part of the table) as described under "Experimental Procedures."

Yeast cells harvested after galactose induction. Each of the cultivations yielded a biomass of about 300 g (wet weight), containing approximately 8000 nmol of P450Cml and 6300 nmol of (Xa)P450Cml.

**Purification and Sequence-specific Proteolysis**—The microsomal (Xa)P450Cml was solubilized with sodium cholate and purified as summarized in Table I. The final preparations were homogeneous in SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 5) and had a specific P450 content of more than 16.1 nmol/mg of protein.

In a next step, the purified (Xa)P450Cml was treated with factor Xa. The major product migrated in SDS-polyacrylamide gel electrophoresis with an apparent molecular weight of 48,000 (Fig. 2, lane 7) compared to 55,000 for the full-length (Xa)P450Cml. As shown after its purification (see below), the 48-kDa protein represented the product of sequence-specific proteolysis at the designed cleavage site and was therefore named Δ(1-66)P450Cml. It was not observed after treatment of the wild-type P450Cml with factor Xa. Under optimized conditions, a cleavage efficiency of up to 70% was achieved and the spectrally detectable amount of P450 remained constant over the incubation period. Most essential additions to the cleavage system were: glycerol (20% final concentration) and DTT (0.5 mM) to prevent P450 denaturation (P420 formation), and detergents to stimulate proteolysis probably by improving the accessibility of the cleavage site. In the absence of any detergents, only about 10% of (Xa)P450Cml were digested by factor Xa. Among several detergents tested (Triton X-100, Tween 20, sodium cholate, and n-dodecyl maltoside), CHAPS was found to give the highest efficiency of the cleavage reaction (data not shown).

**Purification of the Shortened Protein**—The liberated Δ(1-66)P450Cml was separated from the residual uncleaved (Xa)P450Cml by hydrophobic interaction chromatography (Table I). Under the applied conditions, the shortened P450Cml passed unbound through the column, whereas the (Xa)P450Cml was completely retained. The purified Δ(1-66)P450Cml was free of (Xa)P450Cml contaminations, as indicated by SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 8). The NH2-terminal sequence of the purified shortened protein was determined by automated Edman degradation and found to be identical to the sequence of the wild-type P450Cml starting at position Lys-67, confirming that the (Xa)P450Cml was cut exactly after the inserted protease recognition site.

**Molecular Weight of the Shortened P450Cml**—Determination of the molecular weight of purified Δ(1-66)P450Cml by gel filtration on a FPLC column revealed a M, of 90,000, which clearly corresponds to a dimer of the shortened P450Cml. Formation of higher aggregates was not observed. On the other hand, the purified wild-type P450Cml could be eluted under the same conditions with a M, of 470,000, if immediately applied to the column after detergent removal. However, prolonged storage of wild-type P450Cml in the absence of detergent led to the formation of undefined large aggregates.

2. Purification of the cytosolic domain of P450Cml. Microsomal (15 µg) and purified proteins (0.8 µg) were analyzed on a 10% polyacrylamide-SDS gel and visualized with Coomassie Blue. lane 1, molecular weight standards (92,500, 68,000, 45,000, and 29,000); lanes 2 and 3, microsomal fractions of wild-type P450Cml and (Xa)P450Cml, respectively; lane 4, purified P450Cml; lane 5, purified (Xa)P450Cml; lanes 6 and 7, samples of purified P450Cml (lane 6) and (Xa)P450Cml (lane 7) after treatment with factor Xa; lane 8, cytosolic domain 68 (1-66)P450Cml purified on octyl-Sepharose; lane 9, purified NADPH-cytochrome P450 reductase from C. maltosa.

3. Microsomal (Xa)P450Cml is an integral membrane protein and its cytosolic domain can be released by factor Xa treatment. The samples (15 µg of protein) were analyzed by Western blotting using antibodies against P450Cml. Lane 1, untreated microsomes; lane 2 and 3, pellet and supernatant fractions after treatment with sodium carbonate, pH 11.5; lane 4, microsomes after digestion with factor Xa; lanes 5 and 6, pellet and supernatant fractions after ultracentrifugation of factor Xa-digested microsomes. The positions of the starting (Xa)P450Cml, of the cytosolic domain Δ(1-66)P450Cml, and of a minor product of proteolysis with an apparent molecular weight of about 40,000 are marked (*).
Arg-Lys bond (occurring at position 455/456 in P450Cm1) was slowly attacked by factor Xa. This unspecific reaction was almost completely suppressed after optimizing the cleavage conditions with the purified (Xa)P450Cm1 (see Fig. 2).

Spectral and Catalytic Properties—Purified ∆(1-66)-P450Cm1 and wild-type P450Cm1 exhibited identical spectral characteristics with Soret peaks of the oxidized low spin and dithionite-reduced forms at 418 nm and 415 nm, respectively (Fig. 4, A and B), and a reduced CO absorbance maximum at 448 nm (Fig. 4C). In a complete reconstituted system containing purified NADPH-cytochrome P450 reductase and DLPC, the ∆(1-66)P450Cm1 was able to catalyze hexadecane hydroxylation with about 85% of the activity obtained for the wild-type P450Cm1 (Table II). Saturation plots for the interaction of ∆(1-66)P450Cm1, (Xa)P450Cm1, and wild-type P450Cm1 with NADPH-cytochrome P450 reductase are shown in Fig. 5. There were no significant differences between the two membrane-bound P450 forms, indicating that extent of activity and requirement for reductase were not changed upon insertion of the factor Xa recognition site. However, to achieve maximal activities with ∆(1-66)P450Cm1, approximately 2-fold higher amounts of reductase had to be added, compared with P450Cm1. Moreover, there was an almost absolute requirement for lipid in the reconstitution experiments with the shortened P450Cm1. Without DLPC, its enzymatic activity dropped to about 10% (Table II). These results suggest that the functional interaction with NADPH-cytochrome P450 reductase occurred at the lipid surface even after removal of the membrane anchor sequence from the P450 component.

**Table II** Hexadecane hydroxylase activity of purified P450Cm1, (Xa)P450Cm1, and ∆(1-66)P450Cm1 in a reconstituted system

| System | Activity |
|--------|----------|
| P450Cm1 | 3.4 nmol/mm/min/nmol P450 |
| (Xa)P450Cm1 | 3.3 nmol/mm/min/nmol P450 |
| ∆(1-66)P450Cm1 | 2.9 nmol/mm/min/nmol P450 |

Complete system 3.4 3.3 2.9
No DLPC 1.5 1.5 0.3

**DISCUSSION**

Microsomal P450Cm1 was genetically engineered to create a cleavage site for the restriction protease factor Xa immediately after a second hydrophobic segment occurring in its NH₂-terminal region. This modification permitted a sequence-specific proteolysis of the membrane protein and offered the unique chance to study the role of its NH₂-terminal region for membrane-anchoring and functional properties directly at the native membrane-bound protein.

Using intact microsomes, the modified P450Cm1 was found to be accessible to factor Xa digestion, indicating that the designed cleavage site was exposed on the cytosolic surface of the endoplasmic reticulum. The shortened protein produced turned out to be readily soluble even under the mild conditions applied (50 mm Tris/HCl buffer, pH 7.8, 100 mm NaCl) in contrast to the full-length P450, which remained membrane-bound even after treatment with 0.1 mm Na₂CO₃, pH 11.5. This result clearly demonstrates that membrane integration of P450Cm1 was exclusively mediated by the proteolytically removed NH₂-terminal region. Our finding is in agreement with the conclusions on membrane topologies of a number of other eukaryotic P450 forms, which were based on hydrophobicity calculations (6), in vitro translocation experiments with fusion proteins containing P450-derived sequences (25-27) and immunological studies with peptide-specific antibodies (28). However, conflicting results were reported on the expression of truncated P450 proteins in E. coli and yeasts. Whereas in at least one case, P450c7 (29), a mainly cytosolic protein could be obtained, other truncated P450 forms, namely 2E1 (8, 30), 2B4 (8), and 1A1 (31, 32), remained tightly membrane-anchored, suggesting the presence of further determinants for membrane integration in addition to the NH₂-terminal hydrophobic region. This may be due to differences in the structure of the individual P450 forms tested and/or to the differences in expression systems used.

Expression of truncated P450 forms in eukaryotic host/vector systems was generally found to yield rather low levels of functional protein, suggesting that proper folding and stability of P450 proteins may require insertion into the endoplasmic reticulum. This conclusion was substantiated by recent studies on P450Ⅱb17α in COS 1 cells, which demonstrated that both the structure and the catalytic activity are dependent upon the
presence of an amino-terminal sequence that functions as a signal-anchor sequence, and not upon the precise sequence of the amino terminus itself (33). As shown in the present paper with P450Cml, this problem in preparing soluble and functional cytosolic domains of microsomal P450 proteins can be circumvented by expressing the protein in its native membrane-bound state and removing its membrane anchor region in a subsequent step.

The purified Δ(1–66)/P450Cml obtained in large amounts by sequence-specific proteolysis revealed unchanged spectral properties, and its catalytic activity was only slightly decreased compared to the wild-type P450Cml. Therefore, we conclude that, after removal of the NH₂-terminal hydrophobic region from the correctly folded P450 protein, the structure of the cytosolic domain is not significantly affected. This may provide a suitable basis for further studies on structure-function relationships.

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