The N-Terminal Membrane Domain of Yeast NADPH-Cytochrome P450 (CYP) Oxidoreductase Is Not Required for Catalytic Activity in Sterol Biosynthesis or in Reconstitution of CYP Activity*

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K. Venkateswarlu‡, David C. Lamb§, Diane E. Kelly§, Nigel J. Manning¶, and Steven L. Kelly¶

From the Krebs Institute for Biomolecular Research, Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2UH, the §Institute of Biological Sciences, University of Wales Aberystwyth, Aberystwyth SY23 3DA, and the ¶Neonatal Screening Laboratory, Sheffield’s Children’s Hospital, Western Bank, Sheffield S10 2UH, United Kingdom

The disruption of Saccharomyces cerevisiae NADPH-cytochrome P450 oxidoreductase (CPR) gene resulted in a viable strain accumulating approximately 25% of the ergosterol observed in a sterol wild-type parent. The associated phenotypes could be reversed in transformants after expression of native CPR and a mutant lacking the N-terminal 33 amino acids, which localized in the cytosol. This indicated availability of the CPR in each case to function with the monoxygenases squaene epoxidase, CYP51, and CYP61 in the ergosterol biosynthesis pathway. Purification of the cytosolic mutant CPR indicated properties identical to native CPR and an ability to reconstitute ergosterol biosynthesis when added to a cell-free system, as well as to allow reconstitution of activity with purified CYP61, sterol 22-desaturase. This was also observed for purified Candida albicans and human CPR in reconstituted systems. The ability of the yeast enzyme to function in a soluble form differed from human CPR, which is shown to be inactive in reconstituting CYP activity.

NADPH-cytochrome P450 oxidoreductase (CPR)1 (EC 1.6.2.4) is required for microsomal eukaryotic cytochrome P450 (CYP) monoxygenase activity, transferring either both electrons or (sometimes) the first electron for these reactions (1). The CYP enzymes are involved in the metabolism of foreign compounds, such as lipophilic pollutants, pesticides, and drugs, as well as in many biosynthetic reactions (for instance, in steroid, alkaloid, and terpenoid biosynthesis). Although in plants there is CPR diversity, in animal and fungal systems only one CPR has been identified; it functions with the many members of the microsomal P450 superfamily in a particular organism (2). The soluble domain of the enzyme can be generated for structural studies via x-ray crystallography (3, 4), but it was previously reported to be unable to support mammalian CYP activity where the N-terminal membrane anchor was required (5). We are interested in the yeast CPR, and we developed and characterized systems to examine the function of the soluble domain of the enzyme with comparison to human CPR.

We show here, using whole transformants and cell-free systems, that the soluble yeast enzyme can support CYP activity, in contrast to human CPR, which is shown to be inactive.

Within the CYP superfamily, CYP51 is the only family found in animal, plant, and fungal kingdoms, and it represents an ancient metabolic role for CYP in sterol biosynthesis, containing C14 demethylation via three sequential hydroxylations (6). The fungal enzyme is the target for azole antifungals, which are selective in their inhibition over the human and plant orthologues, are central to antifungal chemotherapy, and represent about one-third of the agricultural fungicides used. Two other enzymes of fungal ergosterol biosynthesis require CPR, CYP61 (a sterol 22-desaturase (7–9)), and a non-CYP monoxygenase, squaene epoxidase (10). The former may also be present in plants, in which 22-desaturation is observed, unlike in animals, but the latter is present in all organisms producing sterols (11).

As expected for an antifungal target, gene disruption of CYP51 was observed to be lethal, but the strain could be rescued by providing an ergosterol supplement that could be taken up only anaerobically (12). In contrast, gene disruption of yeast CPR produced viable mutants (13) and ergosterol was present.2 No additional CPR genes could be detected or were subsequently revealed within the yeast genome. In reconstituted assays with purified CPR, enzyme cytochrome b5 can act as alternative donor for the second electron required for monoxygenase activity (14) and may have been supporting catalytic activity in the disruptant. Supporting this concept was the observation that the gene encoding cytochrome b5 can act as a suppressor of a CPR gene disruption phenotype, noted by Sutter and Loper (13), namely hypersensitivity to the CYP51 inhibitor ketoconazole (15). The hypersensitivity suggested that a large reduction in ergosterol biosynthesis might have occurred, making the strain more sensitive, with reduction in ergosterol levels below the critical level occurring at a lower concentration of drug.

Here, we present the first full biochemical characterization of a yeast strain disrupted in the gene encoding CPR, including measurement of activity, enzyme localization, and qualitative and quantitative investigation of sterol profiles. It is shown that an absence of yeast CPR does not result in a dramatic reduction in ergosterol synthesis in the cell. Furthermore, the ability of an N-terminal truncated soluble yeast enzyme to complement a strain containing no CPR and reconstitute CYP activity is demonstrated. This is in direct contrast to genetically engineered soluble human CPR, which is shown to be

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To whom correspondence should be addressed (present address): Institute of Biological Sciences, University of Wales Aberystwyth, Aberystwyth SY23 3DA, United Kingdom. Tel.: 44-1970-622316; Fax: 44-1970-622350.

§ The abbreviations used are: CPR, cytochrome P450 oxidoreductase; CYP, cytochrome P450; PCR, polymerase chain reaction.

2 K. Venkateswarlu, D. C. Lamb, D. E. Kelly, N. J. Manning, and S. L. Kelly, unpublished observation.
inactive, thus revealing a fundamental structural difference between the yeast and mammalian CPR forms.

**EXPERIMENTAL PROCEDURES**

**Strains—**Escherichia coli strain DH5α was used for cloning, and Saccharomyces cerevisiae strain JL20 (MATa, leu2-3, 112, his-4519, ade1-100, ura3-52) was used for expression and gene disruption.

**Construction of Expression Vectors—**The expression vectors containing yeast and human CPR, and the 5′-end truncated CPR forms were constructed in YEplac112, a galactose inducible yeast expression plasmid. Yeast CPR and CPR (Δ33) genes were isolated by PCR using pFBY4 (16) containing yeast CPR as template. The 5′-sense oligonucleotide primers were 5′-CCCGTCGACATCATCATGTCGATGACGGAGATA-TC-3′ for yeast CPR and 5′-CCCGTCGACATCATGC-9′ for human CPR (Δ33) (27) encoded a HindIII site at the 3′-end. The 3′-antisense oligonucleotide primers (5′-CCCAAGCTTTACCAAGCTTTACACCATGTCGATGACGGAGATA-9′) for human CPR (Δ55), 5′-CCCAAGCTTTACCAAGCTTTACACCATGTCGATGACGGAGATA-9′ for CPR (Δ33), and 5′-CCCAAGCTTTACCAAGCTTTACACCATGTCGATGACGGAGATA-9′ for yeast CPR (Δ33) encoded a HindIII site at the 3′-end. The reaction conditions were as follows: 94 °C (denaturation) for 1 min, 45 °C (annealing) for 1 min and 72 °C (extension) for 5 min with 2 min ramp for first 5 cycles and 94 °C for 1 min and 72 °C for 5 min for the remaining 25 cycles in a 30-cycle reaction using Pfu polymerase (Stratagene) and a Perkin-Elmer DNA thermal cycler. Construction of expression vectors for human CPR, a truncated human CPR equivalent to yeast CPR (Δ33) (human (Δ55)), and human CPR digested with trypsin (Δ55) were produced in a similar way using yeast CPR as a template. The 5′-sense oligonucleotide primers (each encoding a SalI restriction site) were 5′-CCCGTCGACATCATGGGACGCTTTACACCATGTCGATGACGGAGATA-TC-3′ and 5′-CCCGTCGACATCATGC-9′ for human CPR, 5′-CCGGTCGACATCATGC-9′ for yeast CPR (Δ33), and 5′-CCCGTCGACATCATGC-9′ for human CPR (Δ55). The 3′-antisense oligonucleotide primer for human CPR, CPR (Δ55), and (Δ55), encoding a HindIII site following the stop codon, was 5′-CCCAAGCTTTACCAAGCTTTACACCATGTCGATGACGGAGATA-9′ for human (Δ33), 5′-CCCAAGCTTTACCAAGCTTTACACCATGTCGATGACGGAGATA-9′ for yeast CPR (Δ33), and 5′-CCCAAGCTTTACCAAGCTTTACACCATGTCGATGACGGAGATA-9′ for yeast CPR (Δ33). PCR conditions for generation of the human CPR fragments were 1 min at 94 °C (denaturation), 1 min and 72 °C (extension) in a 30-cycle reaction using Pfu DNA polymerase. The target fragments were gel purified, digested with SalI and HindIII, and cloned into YEplac112. Transformants were screened by restriction digestion and confirmed by sequencing. All DNA manipulations and transformations were done using standard protocols (17).

**CPR Gene Disruption in JL20—**In the JL20 strain, the chromosomal CPR gene was disrupted by inserting a URA3 to generate the JL20 (CPR::URA3) strain (18). The 0.7-kilobase BamHI internal fragment of the CPR gene in the CPR::YEplac51 construct was replaced by the 1.1-kilobase URA3-containing HindIII fragment isolated from plasmid J4244 (18) by filling in and blunt-ended ligation to obtain a CPR::URA3::YEp51 construct. Chromosomal CPR in the haploid JL20 yeast strain was inactivated using yeast CPR (Δ33) HindIII fragment containing CPR::URA3, and the disruption was confirmed by PCR, using the cells of URA3 colonies of the transformed JL20 strain, the primers and conditions mentioned above (the cells were heated in a microwave for 30 s and quickly cooled on ice for lysis before being subjected to PCR), and ketoconazole susceptibility tests.

**Sterol Isolation and Analysis—**Yeast cells harvested from 100 ml of culture were resuspended in 3 ml of methanol, 2 ml of 60% KOH in water, and 2 ml of 0.5% (w/v) pyrogallol dissolved in methanol and saponified by heating at 90 °C for 1 h. Nonsaponifiable lipids (sterols) were extracted from the saponified mixture three times with 5 ml of ether each time, pooled, and dried under nitrogen. The sterols were suspended in 100 μl of toluene and heated at 60 °C for 1 h for silylation after adding 20 μl of bis(trimethylsilyl) trifluoride. The silyl sterols were analyzed by gas chromatography/mass spectrometry (VG 12–250; VG BIOTECH) by using split injections with a split ratio of 1:3, 2 μl of 1% (w/v) KOH solution was centrifuged at 100,000g for 10 min. The extract was washed with 1000 × g for 15 min to remove mitochondria as a pellet, and then the resulting supernatant was centrifuged at 100,000 × g for 90 min to obtain microsomes as a pellet and cytosol as a supernatant. The microsomal pellet was resuspended in Buffer A using a Potter-Elvehjem homogenizer. Protein content in cell extract and microsomes was measured using the BCA protein estimation kit (Sigma) and bovine serum albumin as a standard (19, 24).

**Purification of Soluble Yeast and Human CPR—**Soluble yeast and human CPR purifications were carried out at 4 °C and a flow rate of 1 ml/min as described previously, with few modifications, and omitting detergent in the purification steps (25). The cytosol of JL20 expressing soluble CPR was precipitated with ammonium sulfate, and the precipitate obtained with 40–65% saturated ammonium sulfate was dissolved in 40 ml of Buffer B (10 mM potassium phosphate buffer, pH 7.0, containing 0.1 μM FAD, 1 μM FMN, 1 mM EDTA, and 10% (w/v) glycerol) and dialyzed overnight against 4 liters of 10 mM Buffer B. The dialyzed solution was centrifuged at 100,000 × g for 60 min to remove precipitated material, and the supernatant was loaded onto a hydroxyapatite column (20 × 10 cm) equilibrated with 10 mM Buffer B. The column was washed with 10 mM Buffer B, and soluble CPR was eluted with a linear concentration gradient of 80 ml of 10–180 mM Buffer B. The soluble CPR-containing fractions were pooled and directly loaded onto a column (1.0 × 8 cm) of 2′,5′-adenosine diphasphate agarose and washed with 100 mM Buffer B, and soluble CPR was eluted with 100 mM Buffer B containing 5 mM adenosine-2′-monophosphate (2′-AMP). The eluate containing soluble CPR was dialyzed overnight against 2 liters of 100 mM Buffer B, concentrated by ultrafiltration, and stored at −80 °C. Native yeast and human CPR were purified according to published procedures (26).

**In Vitro Ergosterol Biosynthesis—**The reaction mixture (1 ml) containing 924 μl of cell-free extract, 50 μl of cofactor solution (1 μmol of NADP, 1 μmol of NAD, 1 μmol of NDPH, 3 μmol of glucose 6-phosphate, 5 μmol of ATP, and 3 μmol of reduced glutathione dissolved in distilled water and adjusted to pH 7.0 with 10M KOH), 15 μl of dilauroyl sodium laurate solution (0.5 μl of n-octane), 5 μl of 0.4M MnCl2, and 10 μl of [3,4-14C]mevalonate (0.25 μCi of 53 mCi/mmol) was incubated at 37 °C and 150 rpm. After 2 h of incubation, the reaction was stopped by adding 1 ml of freshly prepared saponification reagent (15% (w/v) KOH in 90% (v/v) ethanol), and the mixture was saponified by heating at 90 °C for 1 h. The nonsaponified sterols were extracted from the mixture twice with 3 ml of petroleum ether (boiling point 40–60 °C). The extract was dried, re-dissolved in 100 μl of petroleum ether. The nonsaponifiable sterols were applied to silica gel thin layer chromatography plates (ART 573, Merck) and developed using toluene:diethyl ether at a ratio of 9:1 (v/v). Radioactive sterols were localized by autoradiogram and excised for scintillation counting (27).

**Reconstitution of CYP Activity—**The standard reaction mixtures contained purified S. cerevisiae CYP61, C. albicans and human CYP51 (0.5 nmol), 1 unit of either native yeast and human CPR or the N-terminal truncated soluble CPR forms, 13 nmol of the respective substrate (ergosta-5,7-dienol for S. cerevisiae CYP61 and dihydrostanol or 24-methylene-24, 25-dihydrostanol for human and C. albicans CYP51, respectively) dispersed in 90 nmol dilauroylphosphatidylcholine; the reaction volume was adjusted to 950 μl with 100 mM potassium phosphate buffer, pH 7.2. NADPH was added at a concentration of 1 mM to the mixture to start the reaction. C. albicans CYP51 was purified as described previously (28), and the human CYP51 was purified after GAL10 expression in yeast (29). All reactions were incubated at 37 °C for 20 min in a shaking water bath. Reactions were stopped by the addition of 5 ml of methanol, and the mixture was extracted with 1 ml of ethyl acetate. The extracts were analyzed by gas chromatography/mass spectrometry as described above. Trimethylsilylated derivatives of sterol substrates and metabolites were clearly separated as two distinct peaks (for example, see Ref. 9). The conversion

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3 D. C. Lamb, D. E. Kelly, M. Akhtar, M. R. Waterman, M. Stromstadt, D. Rozman, and S. L. Kelly, submitted for publication.
N-Terminal Domain of CPR Not Required for Catalytic Activity

**RESULTS**

**Complementation Studies Using a cpr- Gene Disruptant**—The gene-disrupted strain (CPR::URA3/JL20) was generated as described above and had the phenotype described previously for a disruption using LEU2 in the same way (13). Deletion of the CPR gene led to a decrease in the levels of ergosterol. The ergosterol content in the cpr-disrupted strain was 4-fold lower than that of the undisrupted strain (JL20). However, ergosterol was the major sterol in both the strains and accounted for about 90% of the total sterols. In the cpr host strain, the ergosterol levels were restored to the levels in the CPR strain JL20 upon yeast CPR and CPR (Δ33) expression (Table I). Expression of these proteins in JL20 (CPR) did not alter the ergosterol content (data not shown).

**Expression of Yeast and Human CPR Proteins in the Transformed Strains**—For Western blot analysis of the expression and localization of both yeast CPR and CPR (Δ33), the cytochrome c reductase activity was measured as described previously (29). The absorption spectra of the purified yeast CPR (Δ33) were obtained by scanning from 300 to 700 nm (4). The ergosterol content in the non-ya

**TABLE I**

| Sterols were extracted as described under “Experimental Procedures” and analyzed by gas chromatography/mass spectrometry. |
|---|
| **Transcript** | **Ergosterol** | **Dry weight of cells** | **Total sterols** |
| **mg/g** | **%** | **%** |
| JL20 | 19.7 ± 2.2 | 89.0 ± 4.8 |
| JL20 (CPR::URA3) | 5.9 ± 0.3 | 89.3 ± 3.7 |
| YEp51/JL20 (CPR::URA3) | 5.5 ± 0.6 | 89.3 ± 5.6 |
| CPR (Δ33)/YEp51/JL20 (CPR::URA3) | 17.4 ± 1.6 | 91.9 ± 4.3 |
| CPR::URA3 | 21.2 ± 3.3 | 86.4 ± 5.5 |

**Spectrophotometric Measurements**—A Philips PU8800 UVVIS scanning spectrophotometer was used for all spectral studies. Cytochrome c reductase activity was measured as described previously (29). The absorption spectra of the purified yeast CPR (Δ33) were obtained by scanning from 300 to 700 nm (4). The ergosterol content in the non-yeast or human native CPR protein. However, in transfectants expressing soluble yeast and human CPR in the CPR host JL20, the enzyme was observed in the cytosol as well as in the microsomes. The amount of CPR present in the soluble CPR-expressing microsomes was quite similar to that of microsomes from transformants containing the empty vector, YEp51, representing the basal endogenous CPR level in the strain JL20. In addition, no CPR was detected in the microsomal fractions of the CPR strain transformed with either YEp51 or CPR (Δ33); YEp51 after induction of expression, but it was observed in the cytosolic fractions. The CPR level found in the cytosol was the same as that observed in the cytosol of CPR strain expressing CPR (Δ33)/YEp51 (data not shown). These results clearly indicated that the N-terminal truncated yeast and human CPR localized in cytosol after expression similar to the previous reports following trypsin cleavage experiments on mammalian CPR (3, 4).

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**Purification of Soluble Truncated CPR**—Purification of yeast CPR (Δ33) to homogeneity by standard CPR methodology of ammonium sulfate fractionation, hydroxyapatite, and 2'-adenosine diphosphate agarose (affinity) chromatography yielded a 75-kDa protein. The purified yeast CPR (Δ33) reduced cytochrome c at a rate of 24.3 nmol/min/mg of protein, compared with 36 μmol/min/mg of protein for human CPR (Δ50) and CPR (Δ55). From this activity, the CPR activity was estimated as 8 and 12 nmol/min/mg of protein for yeast and human truncated CPR by assuming 1 nmol CPR reduces 3 nmol of flavin cofactors (Fig. 2). The oxidized and reduced spectra of purified yeast CPR (Δ33) protein were recorded to confirm the presence of flavin cofactors (Fig. 2). The oxidized spectrum of yeast CPR (Δ33) had peaks at 380 and 455 nm. The spectrum obtained with the NADPH reduced CPR (Δ33) showed a decrease in absorbance at 455 nm, an increase in absorbance at 380 nm, and a broad absorption band between 550 and 650 nm characteristic of the air-stable semiquinone form. These spectra were identical to those reported for native microsomal yeast CPR (4).

**Catalytic Activities of Yeast CPR in Sterol Biosynthesis**—In vitro ergosterol biosynthesis was carried out by using the cell extracts of the various transformants of the CPR host (Table III), an assay applied for assessment of ergosterol biosynthesis inhibitors. The amount of ergosterol synthesized in vitro was reduced by about 3-fold upon CPR disruption when compared
Wavelength (nm)

**FIG. 2.** The absorption spectra of purified yeast CPR (Δ33) enzyme (6 μM). —— oxidized form; - - - - the semiquinone (reduced) form was prepared by adding NADPH to a final concentration of 200 μM and letting the sample be equilibrated for 10 min at room temperature.

**TABLE III**

| Transformant | In vitro ergosterol biosynthesis | Ergosterol | IC₅₀ |
|--------------|----------------------------------|------------|------|
| JL20         | ND                               | 8950 ± 429 | 22.12 ± 3.73 |
| JL20 (CPR::URA3) | ND                               | 3345 ± 324 | 0.11 ± 0.06 |
| YEp51/JL20 (CPR::URA3) | ND                               | 3178 ± 648 | 0.12 ± 0.04 |
| CPR (Δ33)/YEp51/JL20 (CPR::URA3) | ND                               | 8134 ± 374 | 14.78 ± 2.98 |
| YEp51/JL20 (CPR::URA3) + CPR (Δ33) | ND | 9256 ± 492 | 25.49 ± 4.67 |
| YEp51/JL20 (CPR::URA3) + CPR (Δ33) | ND | 7246 ± 521 | 11.97 ± 3.28 |
| YEp51/JL20 (CPR::URA3) + CPR (Δ33) | ND | 7679 ± 712 | 13.88 ± 5.13 |

* Purified yeast CPR (Δ33) equivalent to the amount of CPR present in JL20 was added.

**TABLE IV**

| Transformant | Turnover (min⁻¹) |
|--------------|-----------------|
| Yeast CPR51  | 0.78 ± 0.10     |
| C. albicans | 0.104 ± 0.13    |
| Human CPR51 | 0.12 ± 0.09     |

We confirmed the observation of Sutter and Loper (13) of a 200-fold increased sensitivity in such a strain to ketoconazole, but we observed only a relatively small reduction in ergosterol synthesized to about 25% of the parent strain when compared by dry weight of cells. Analysis of sterols by gas chromatography and thin layer chromatography did not reveal accumulation of intermediates, such as lanosterol or ergosta-5,7-dienol, that are indicative of a block to enzyme activity at the steps in which CPR participates. This indicated that the electron donor system remaining in the cells is efficient, delivering both electrons for CYP activity.

Previously, the N terminus of mammalian CPR has been indicated to be the membrane anchor for the protein because tryptic cleavage was observed to release a soluble domain (5). However, this was not catalytically active in reconstituted systems, suggesting a role for the N terminus in interactions with CYP. Our studies indicate that the N-terminal 33 amino acids are important for membrane anchoring and targeting for yeast CPR, which contains a similar hydrophobic sequence. In contrast to previous studies on mammalian enzyme, this cytosolic protein is catalytically active and complements cpr-disrupted sterol biosynthesis on both expression and addition of purified enzyme to the in vitro system. This observation was confirmed in reconstitution studies with CYP61, in which normal activity was observed, as well as studies with C. albicans and human CPR51. This difference from mammalian studies was confirmed for the human enzyme and reflects a fundamental difference between the CPRs from different kingdoms. We can conclude that the yeast CPR membrane anchor is not required for supporting P450 activity in vivo or in vitro. Recently, the structure of rat CPR was resolved at 2.6 Å (31), and similar resolution of the yeast enzyme structure may help to elucidate the fundamental differences between the enzymes. For crystallization, the rat protein was produced in its soluble form after tryptic digestion, and the manner in which the hydrophobic domain allows interaction with CYP enzymes remains unclear.

Presumably, the hydrophobic domain is important for the correct spatial interaction to allow electron transfer to CYP enzymes. Such interactions appear unimportant for yeast CPR in its interaction with fungal and mammalian CYP forms, and we are currently undertaking crystallization trials on soluble yeast CPR to resolve this and other issues.

**DISCUSSION**

One of the earliest roles of CPR, as for the CYP superfamily, may have been in sterol biosynthesis. CPR also functions in the squalene epoxidation reaction (10), which precedes sterol 14α-demethylation undertaken by CYP51 (6), as well as sterol 22-desaturation undertaken by CYP61, one of the last steps in the ergosterol biosynthesis pathway (30).

The previous finding of hypersensitivity to ketoconazole on disruption of the gene encoding CPR was in many ways consistent with the concept of inefficient sterol biosynthesis, so that only a low dose of CYP51 inhibitor would arrest growth.

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