Biodiversity of the P450 catalytic cycle: yeast cytochrome b_5/NADH cytochrome b_5 reductase complex efficiently drives the entire sterol 14-demethylation (CYP51) reaction

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Abstract The widely accepted catalytic cycle of cytochromes P450 (CYP) involves the electron transfer from NADPH cytochrome P450 reductase (CPR), with a potential for second electron donation from the microsomal cytochrome b_5/NADH cytochrome b_5 reductase system. The latter system only supported CYP reactions inefficiently. Using purified proteins including *Candida albicans* CYP51 and yeast NADPH cytochrome P450 reductase, cytochrome b_5 and NADH cytochrome b_5 reductase, we show here that fungal CYP51 mediated sterol 14α-demethylation can be wholly and efficiently supported by the cytochrome b_5/NADH cytochrome b_5 reductase electron transport system. This alternative catalytic cycle, where both the first and second electrons were donated via the NADH cytochrome b_5 electron transport system, can account for the continued ergosterol production seen in yeast strains containing a disruption of the gene encoding CPR.

Key words: P450 catalytic cycle; Cytochrome P450 reductase; Yeast; *Candida albicans*

1. Introduction

Cytochromes P450 (CYPs) are haem-thiolate proteins involved in the oxidative metabolism of endogenous and xenobiotic compounds [1,2]. The post-genomic era is revealing a huge number of such proteins that can be involved in biosynthetic reactions in steroid, prostaglandin, secondary metabolism and others. Most attention to date has been placed on mammalian enzymes involved in xenobiotic metabolism, particularly as they are central to the metabolic fate of pharmaceuticals, chemical carcinogens and environmental organic pollutants [3].

Cytochromes P450 are not self-sufficient enzymes and, for function, eukaryotic microsomal forms require NADPH cytochrome P450 reductase (CPR) as an electron donor [4]. In early investigations of the mammalian electron transport system, and of the P450 catalytic cycle, key features were established [5–9]. To perform catalysis, P450 must receive two electrons from NADPH via CPR. Cytochrome b_5 has been implicated as a component in this electron transfer cycle for some cytochromes P450, being capable/involved in the transfer of the second electron to P450, but not the first (Fig. 1). For some cytochromes P450 a higher activity results from participation of cytochrome b_5 [10,11], while for CYP17 cytochrome b_5 can also act to alter the reaction undertaken by this cytochrome P450 form suggesting a modulatory effect on the CYP active site might also occur [12,13]. The cytochrome b_5/NADH cytochrome b_5 reductase electron transport pathway is also involved in other non-CYP reactions of sterol biosynthesis and in fatty acid metabolism [14].

Within the yeast *Saccharomyces cerevisiae* genome, only three CYP genes were revealed which require CPR for function: CYP51, sterol 14α-demethylase, undertaking C14-demethylation of lanosterol; CYP61, sterol 22-desaturase, undertaking C22-desaturation of ergosta-5,7-dienol (both involved in the ergosterol biosynthetic pathway); and CYP56, dityrosine hydroxylase, involved in yeast sporulation [15]. Additionally, a further enzyme of the sterol biosynthetic pathway requires CPR, namely squalene epoxidase [16]. Yeast gene disruption studies on CPR indicated surprisingly that the protein was dispensible [17,18] and that ergosterol was the predominant sterol with or without CPR being present in strains [17,19]. With possession of the full genome it was evident that no further CPR gene sequences are present to compensate and confer functional redundancy. Here we address directly the potential of the yeast cytochrome b_5/NADPH cytochrome b_5 reductase system to drive CYP51 using heterologous expression, purification and reconstitution of enzyme activity. Biodiversity of the CYP catalytic system was indicated as the yeast cytochrome b_5/cytochrome b_5 reductase system and supported the catalytic cycle entirely and as efficiently as CPR, unlike the observations made previously with mammalian enzymes.

2. Materials and methods

2.1. Cloning and heterologous expression of yeast cytochrome b_5 and NADH cytochrome b_5 reductase

The GeneStorm yORF expression vectors pYES2/gs containing the *S. cerevisiae* cytochrome b_5 and NADH cytochrome b_5 reductase genes were obtained from Invitrogen and used as a source of recombinant protein. The gene insert in each GeneStorm yORF expression vector was PCR amplified, the fragments correctly sized and the orientation verified. Each expression plasmid was transformed into competent *Escherichia coli* strain DH5α with transformants selected on LB plates containing 50 μg/ml ampicillin. Following bacterial transformation and isolation of plasmid DNA, expression vectors were transformed into a host yeast strain, BY4743 (MATa/α *leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 his3Δ0/hisΔ0 LYS2/lys2 MET15/met15Δ0*) using

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published procedures [20]. Yeast transformants were grown at 28°C, 250 rpm with 250 ml culture in 500 ml flasks. The media used consisted of Difco yeast nitrogen base without amino acids supplemented with 100 mg/l histidine and 100 mg/l leucine and 2% (w/v) glucose as initial carbon source. Heterologous expression was induced when the glucose was exhausted at a cell density of approximately 10^8 cells/ml. The culture was left a further 4 h before galactose was added to a final concentration of 3% (w/v). After 20 h induction, cells were harvested by centrifugation, resuspended in buffer containing 0.4 M sorbitol, 50 mM Tris-HCl pH 7.4 and broken using a Braun disintegrator (Braun GmbH, Mesungen, Germany) with four bursts of 30 s together with cooling from liquid carbon dioxide. Cell debris was removed by centrifugation at $1500 \times g$ for 10 min using a MSE bench centrifuge. The resulting supernatant was centrifuged twice at $10\,000 \times g$ for 20 min to remove mitochondria and finally at $100\,000 \times g$ for 90 min to yield the microsomal fraction. This was resuspended using a Potter-Elvehjeim glass homogeniser at about 10 mg protein/ml in the same buffer described above.

2.2. Purification of yeast cytochrome b_5 and NADH cytochrome b_5 reductase

The microsomal fractions prepared from cells heterologously expressing each protein were solubilised in 100 mM potassium phosphate buffer, pH 7.4 containing 2% (w/v) sodium cholate for 1 h at 4°C. Solubilised proteins was recovered after a 1 h centrifugation step at $100\,000 \times g$ and diluted with a 20% (v/v) glycerol/NaCl solution to 25 mM potassium phosphate, 500 mM NaCl, 0.8% (w/v) sodium cholate. The supernatant was applied directly to a ProBond resin column (Invitrogen) previously equilibrated with a 25 mM potassium phosphate buffer, pH 7.4 containing 500 mM NaCl and 0.8% (w/v) sodium cholate. ProBond resin column allows the purification of recombinant proteins expressed so as to contain a C-terminal polyhistidine sequence. The protein was recovered from the crude solubilised microsomal fractions by high affinity binding to divalent nickel ions on the resin. The column was washed with equilibration buffer and slowly eluted with the same wash buffer, but containing 300 mM imidazole. Fractions containing cytochrome b_5 and NADH cytochrome b_5 reductase were concentrated and stored at −80°C until use.

2.3. Characterisation of cytochrome b_5 and NADH cytochrome b_5 reductase expression

Analysis of cytochrome b_5 and NADH cytochrome b_5 reductase protein levels was made by immunoblotting. Purified proteins were separated by electrophoresis on 12% gels (w/v) according to the method of Laemmli [21] and transferred to nitrocellulose. Detection of recombinant proteins was determined using the anti-V5-HRP (horseradish peroxidase conjugated) antibody, which allows detection of recombinant proteins containing the C-terminal V5 epitope [22]. The anti-V5-HRP antibody recognises the sequence -Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr-. Immunodetection of cytochrome b_5 and NADH cytochrome b_5 reductase was performed using chloronaphthol as described previously [23].

2.4. Expression and purification of recombinant Candida albicans sterol 14α-demethylase

Yeast transformants were cultivated in yeast minimal medium containing 1.34% (w/v) Difco yeast nitrogen base without amino acids, 100 mg/l L-histidine and 2% (w/v) glucose as initial carbon source. Heterologous protein expression was induced after exhaustion of glucose in the medium by addition of galactose to a final concentration of 3% (w/v). After 24 h induction cells were harvested by centrifugation and mechanically disrupted [24]. Microsomes were obtained following $100\,000 \times g$ ultracentrifugation and the expressed CYP51 was solubilised with 2% (w/v) sodium cholate and purified on ω-amino-*n*-octyl Sepharose 4B and hydroxyapatite as previously described [25].

2.5. Expression and purification of recombinant S. cerevisiae cytochrome P450 reductase

S. cerevisiae JL20 transformants carrying native yeast CPR expression vector was grown in yeast minimal medium containing Difco yeast nitrogen base without amino acids (1.34%, w/v), 100 mg/l L-histidine and glucose (2%, w/v) at 30°C until glucose was completely consumed and then heterologous expression was induced with galactose (3%, w/v) for 20 h [19,26]. Cells were harvested by centrifugation and subjected to mechanical breakage. The microsomal component, containing full length CPR, was isolated by ultracentrifugation. Purification of yeast CPR was carried out according to published procedures [27].

2.6. Reconstitution of CYP51 activity with CPR and/or cytochrome b_5 and NADH cytochrome b_5 reductase

Each reaction mixture contained purified CYP51 (0.1 nmol) and the indicated reductase system at equimolar concentrations (P450 reductase or cytochrome b_5/NADH cytochrome b_5 reductase) in a total volume of 50 μl. For reconstitution studies, 23 nmol of substrate, 24-methylene-24,25-dihhydrolanosterol, was dispersed in 50 μg dilauroylphosphatidylcholine (DLPC), sonicated until a white suspension had formed and dried down under nitrogen. The mixture was redissolved with the enzymatic components of the reactions and NADPH was added at a final concentration of 1 mM to the mixture to start the reaction. All reactions were incubated at 37°C and were terminated by the addition of 5 ml methanol. Sterol substrate and metabolite were extracted and analysed by gas chromatography/mass spectroscopy [28].

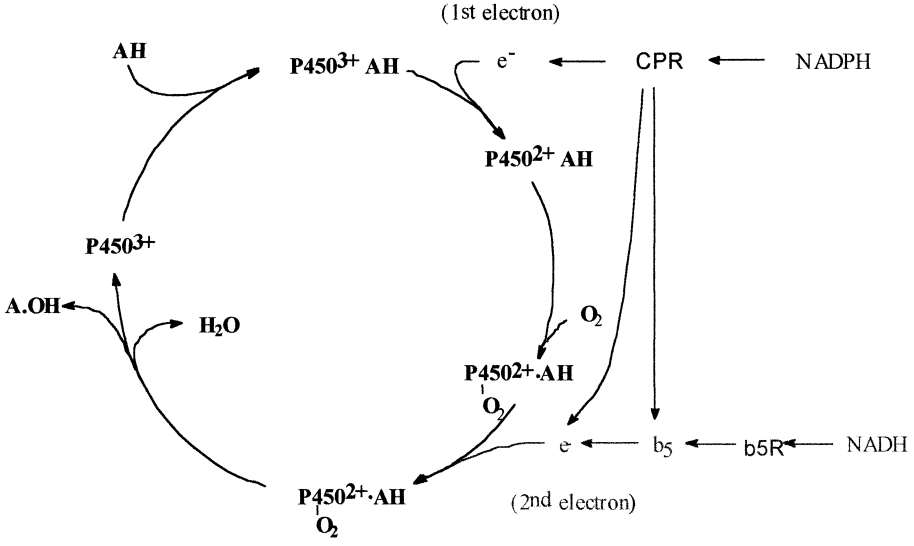

Fig. 1. The catalytic cycle of cytochrome P450. NADPH cytochrome P450 reductase (CPR) supplies two electrons to P450 for catalysis via NADPH. Cytochrome b_5/NADH cytochrome b_5 reductase complex has been implicated as a component in this electron transfer cycle for some cytochromes P450, being involved in the transfer of the second electron to P450, but not the first. The catalytic cycle is initiated by binding of the substrate (AH) to the oxidised cytochrome ($P450^{3+}$).

3. Results

3.1. Heterologous expression and purification of S. cerevisiae cytochrome b_5 and NADH cytochrome b_5 reductase

In order to investigate the role of *S. cerevisiae* cytochrome b_5 and NADH cytochrome b_5 reductase in supporting CYP51 activity, GAL1 mediated heterologous expression was undertaken using the yeast vector pYES2/gs containing both open reading frames (ORFs) (obtained from the Genestorm collection of Invitrogen). The microsomal fractions prepared from yeast expressing these proteins were probed with the anti-V5 antibody. The mobilities of the expressed proteins were higher than their predicted molecular masses (14 kDa for cytochrome b_5 and 34 kDa for NADH cytochrome b_5 reductase) as expected due to the presence of the C-terminal V5 epitope and poly-His-tag (18 kDa for cytochrome b_5 and 38 kDa for NADH cytochrome b_5 reductase). Western blots also revealed that both proteins were expressed exclusively in the microsomal fractions of the yeast as expected (data not shown). Purification of both recombinant cytochrome b_5 and NADH cytochrome b_5 reductase to homogeneity was achieved following immobilised metal affinity chromatography using Pro-Bond resin, which utilises the metal binding domain encoded by the polyhistidine tag. Both recombinant cytochrome b_5 and NADH cytochrome b_5 reductase were solubilised in 2% (w/v) sodium cholate and affinity purified with the aid of the C-terminal His-tag using an Ni-nitrilotriacetic acid agarose column. Recombinant cytochrome b_5 reductase showed the absolute absorption spectra characteristic of flavoproteins. The oxidised form showed prominent peaks at 460 and 390 nm, typical of a flavoprotein (Fig. 2A), and the 460 nm peak disappeared when reduced by 100 μM NADH. These results are typical of native cytochrome b_5 reductase proteins purified from other organisms [29,30]. The recombinant cytochrome b_5 showed an absorption maximum at 413 nm (Fig. 2B), and the dithionite-reduced form exhibited prominent peaks at 424, 526 and 557 nm. These spectral characteristics are typical of the

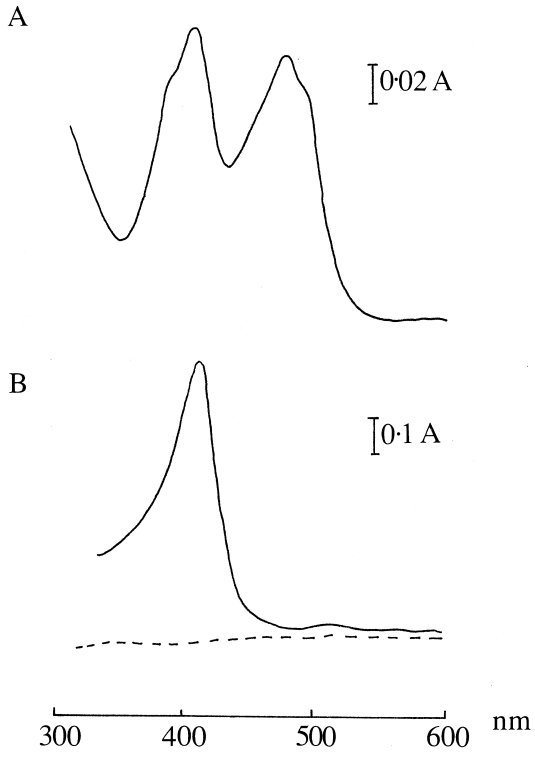

Fig. 2. A: Absolute absorption spectrum of purified, recombinant yeast NADH cytochrome b_5 reductase. Purified NADH cytochrome b_5 reductase, 0.2 nmol, was dissolved in 100 mM potassium phosphate buffer, pH 7.2, and the absolute spectra were recorded (solid line, oxidised form). B: Absolute absorption spectrum of purified, recombinant yeast cytochrome b_5. Purified cytochrome b_5, 0.2 nmol, was dissolved in 100 mM potassium phosphate buffer, pH 7.2, and the absolute spectra were recorded (solid line, oxidised form). Dashed line represents the spectrum obtained with yeast microsomes expressing the null vector.

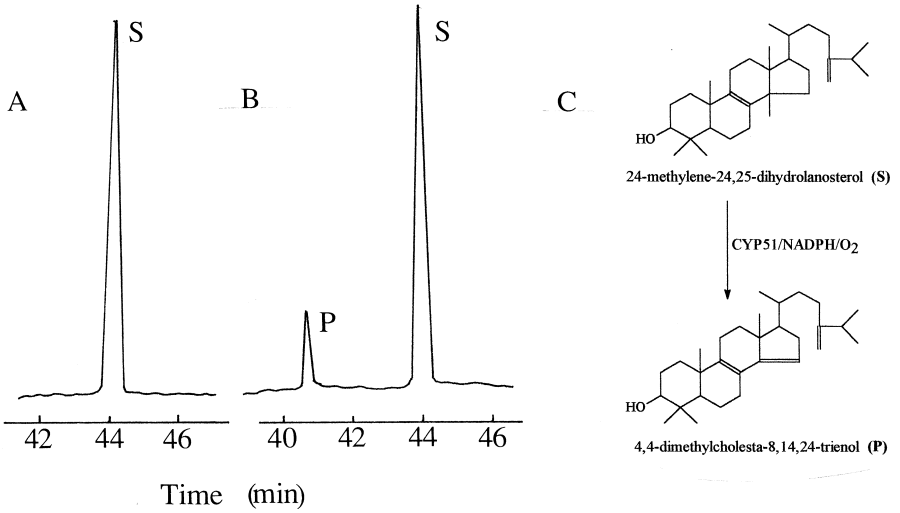

Fig. 3. Catalytic activities of purified *C. albicans* CYP51 driven by cytochrome b_5/NADH cytochrome b_5 reductase complex. A: Gas chromatogram of 24-methylene-24,25-dihydrolanosterol (**S**) with a retention time located at 44 min. B: The conversion of 24-methylene-24,25-dihydrolanosterol (**S**, retention time 44 min) to 4,4-dimethylergosta-8,14,24(25)-trienol (**P**, retention time 40.8 min) catalysed by *C. albicans* CYP51 reconstituted with cytochrome b_5/NADH cytochrome b_5 reductase complex as monitored by gas chromatography/mass spectroscopy outlined in Section 2. C: 24-Methylene-24,25-dihydrolanosterol 14α-demethylation. Conversion of 24-methylene-24,25-dihydrolanosterol to 4,4-dimethylergosta-8,14,24(25)-trienol in the presence of *C. albicans* CYP51, NADPH and molecular oxygen.

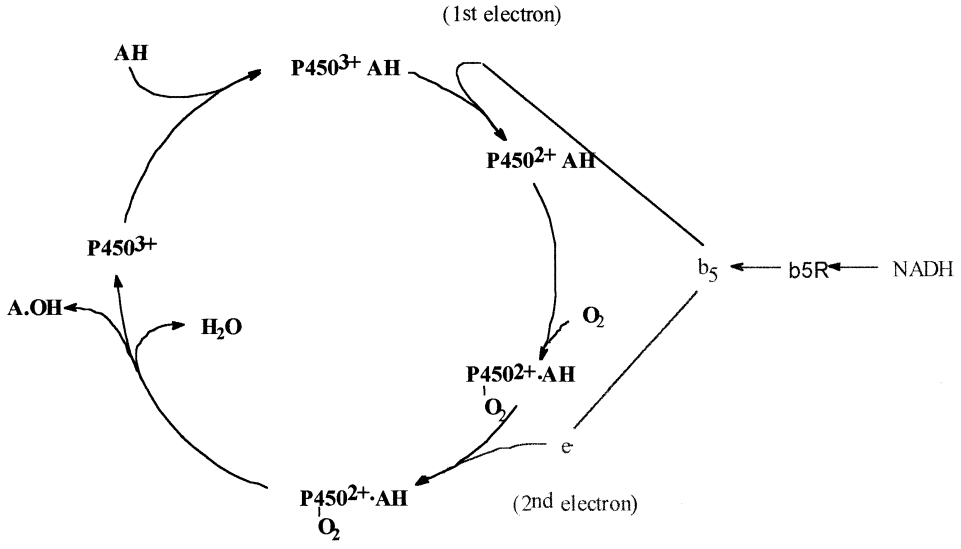

Fig. 4. An alternative catalytic cycle for cytochrome P450 in yeast. The results of the present study reveal yeast CYP mediated activity supported wholly by the cytochrome b_5/NADH cytochrome b_5 electron transport system. A fundamental difference from the original framework elucidated for mammalian CYP catalytic cycle is established.

native cytochrome b_5 proteins purified from other sources [31,32].

When the oxidised form of the recombinant cytochrome b_5 was incubated with the recombinant cytochrome b_5 reductase protein and 100 μM NADH, it was rapidly reduced and showed an absolute absorption spectrum similar to that of the dithionite-reduced form with a spectral maximum at 424 nm. The recombinant NADH cytochrome b_5 reductase was functionally active. No reduction of cytochrome b_5 was observed in the presence of NADPH and cytochrome b_5 reductase, demonstrating that cytochrome b_5 reductase did not transfer the reducing equivalents from NADPH to cytochrome b_5.

3.2. *Catalytic activity studies on sterol 14α-demethylase with cytochrome b_5, NADH cytochrome b_5 reductase and NADPH cytochrome P450 reductase (CPR)*

Previously, we have reported that disruption of *S. cerevisiae* NADPH cytochrome P450 reductase (*CPR*) gene resulted in a strain accumulating ergosterol [19]. This indicated that the cytochrome P450 sterol biosynthetic enzymes, namely sterol 14α-demethylase (CYP51) and sterol Δ^{22}-desaturase (CYP61), could receive electrons to utilise in their catalytic reaction mechanisms from other electron donor proteins other than CPR. To investigate if cytochrome b_5/NADH cytochrome b_5 reductase might be responsible we reconstituted with purified *C. albicans* CYP51 to determine activity. A reconstituted system containing CYP51, cytochrome b_5 and NADH cytochrome b_5 reductase could catalyse the 14α-demethylation of 24-methylene-24,25-dihydrolanosterol to 4,4-dimethylcholesta-8,14,24(28)-trienol (Fig. 3). CYP51, receiving reducing equivalents via the cytochrome b_5/NADH cytochrome b_5 reductase complex, could catalyse sterol 14-demethylation of 24-methylene-24,25-dihydrolanosterol with similar activity to CPR mediated CYP51 sterol demethylation. Turnover numbers were comparable to those for CPR driven sterol demethylation. For CYP51+CPR reactions a K_M of 21 and V_{MAX} of 0.24 ± 0.04 were observed compared to CYP51+CYT b_5+CYT b_5 reductase reactions where a K_M of 21 and V_{MAX} of 0.19 ± 0.03 were found. K_M was expressed as μM and V_{MAX} as nmol product formed/min/nmol *C. albicans* CYP51. These values were similar to other reports on reconstituting the activity of this CYP [25,26]. When using rat cytochrome b_5 in place of the yeast enzyme, activity was below the level of detection using this assay. In experiments, values between 0.2–0.3 nmol product/min/nmol CYP51 were obtained where within experiment variation between CPR driven and cytochrome b_5/NADH cytochrome b_5 reductase driven values for enzyme turnover was observed to be approximately 20%.

4. Discussion

Since the discovery of the cytochromes P450 and the first characterisation of the systems during the 1960s, a role for CPR as the electron donor reductase was recognised and reconstitution of activity was achieved [5,6]. Early studies indicated simultaneous addition of NADH could stimulate NADPH-dependent microsomal monooxygenase activity [33]. This NADH effect was proposed to indicate a possible involvement of cytochrome b_5 and the NADH electron transfer pathway in providing the second electron needed for monooxygenase activity [34]. Further support for this belief was obtained from immunological studies utilising anti-cytochrome b_5 antibodies [35,36] and the NADH electron transfer pathway has only been observed to support CYP activity on its own at sub-optimal levels. This model has been accepted as a central belief regarding the CYP catalytic cycle, although for some mammalian CYP forms no stimulation of activity has been observed and also for particular substrates of certain isoforms. In some CYP reactions cytochrome b_5 has been reported as an obligatory component [37], including the important drug metabolising enzyme, human liver CYP3A4 [11]. The presence of cytochrome b_5 can also alter the type of chemical reaction undertaken by CYP17 from 17-hydroxylation to the lyase reaction [12,13].

Yeast sterol biosynthesis is the most complete sterol pathway for which a gene complement is available. Besides being a model system for sterol studies it is also important as the target of the commercially important ergosterol biosynthesis inhibitors, which include the azole antifungal CYP51 inhibitors, and for metabolic engineering studies to produce vitamin D and steroids. The disruption of the gene encoding yeast CPR (*NCPR1* in yeast nomenclature) was first undertaken and reported by Sutter and Loper [17]. Strains were viable, and could synthesise ergosterol as the predominant sterol [19]. Clearly an efficient electron donor system existed to support CYP51 (sterol 14-demethylase; Erg11p) and CYP61 (sterol 22-desaturase, Erg5p) catalytic activity, as well as squalene epoxidase (Erg1p), in the absence of further CPRs within the yeast genome. Further evidence that cytochrome b_5 might be important in this role came from observations that a double disruption of cytochrome b_5 and CPR was lethal to the cell [18]. The cytochrome b_5/NADH cytochrome b_5 electron transport system also participates in other sterol biosynthetic reactions such as C5-desaturation and C4-demethylation [38,39]

We have addressed these issues using purified components of the yeast NADPH-CPR and NADH cytochrome b_5 electron transfer systems to support *C. albicans* CYP51 mediated activity. We used GAL10- and GAL1-based yeast expression systems to produce CYP51 and CPR *and* cytochrome b_5 and NADH cytochrome b_5 reductase, respectively. Expression of CYP51 and CPR was as reported previously. The GAL1-based GeneStorm system expressed cytochrome b_5 and NADH cytochrome b_5 reductase to high levels of >5 mg recombinant protein/l culture respectively, reflecting an efficient system for producing such membrane-bound enzymes. This included the C-terminal addition of a V5 epitope for immunological recognition and the His-tag for efficient purification. After purification addition of the proteins in reconstitutions for CYP51 activity was undertaken with comparison to CPR supported activity. Our findings of efficient CYP mediated activity supported by the cytochrome b_5/NADH cytochrome b_5 electron transport system indicate biodiversity from the original framework elucidated for mammalian CYP catalytic cycle where the second electrons could be derived from cytochrome b_5. A summary of this alternative electron transfer cycle is outlined in Fig. 4.

Why such biodiversity exists deserves further consideration with regard to whether the mammalian information is applicable for all organisms. No data from plants at present have indicated the cytochrome b_5/NADH cytochrome b_5 reductase system can support CYP activity, although cytochrome b_5 appears important in stimulating some CYP activities [40]. In *Mycobacteria* a soluble CYP51 exists, probably supported by flavodoxin/flavodoxin reductase systems, but the relationship of this novel pathway of cholesterol biosynthesis to eukaryotes is unclear, although intriguing [41]. Within the ergosterol biosynthetic pathway the continued production of sterol in CPR gene disrupted strains was observed previously [17,19]. The results presented here explain this observation and reveal interchangability in yeast electron transport donor for CYP51 and by extension to CYP61 and squalene epoxidase also. The slight reduction in sterol content for the CPR gene disrupted yeast strains analysed before might be due to increased burden on the cytochrome b_5/NADH cytochrome b_5 electron transport system. We discussed previously the likely evolution of CYP61 from CYP51, and the ability of the former to metabolise xenobiotics [28,42], and despite the changes in reaction mechanism involved (14-demethylation to 22-desaturation) retention of the catalytic requirements for CYP61 is likely to have been maintained from CYP51. It is most likely this alternative catalytic cycle will hold for fungal sterol biosynthesis generally. A similar catalytic cycle cannot be excluded elsewhere throughout the Kingdoms of Life.

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