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Characterization of *Saccharomyces cerevisiae* CYP51 and a CYP51 Fusion Protein with NADPH Cytochrome P-450 Oxidoreductase Expressed in *Escherichia coli*

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***Saccharomyces cerevisiae* CYP51, target of azole antifungal agents, and CYP51 fused with *S. cerevisiae* cytochrome P-450 oxidoreductase (FUS protein) were expressed in active forms in *Escherichia coli* by cloning into pET15b. The expression was monitored immunologically, catalytically, and by using reduced carbon monoxide difference and type II binding spectra. CYP51 and FUS enzymes were located in membranes and produced a Soret peak at 448 nm in the reduced CO difference spectrum. The cytochrome P-450 contents in the membrane fractions containing CYP51 and FUS proteins were 12.8 ± 2.6 and 17.4 ± 3.7 pmol/mg of protein, respectively. The NADPH cytochrome P-450 oxidoreductase (CPR) content was estimated to be 15.7 ± 1.1 pmol/mg of protein in FUS membrane fractions. FUS protein catalyzed the demethylation of substrate at the 14α position, with a turnover number of 1.96 ± 0.37 min^{-1} in the presence of NADPH. No reductase activity was observed in membrane fractions containing CYP51, and therefore, CYP51 did not function catalytically in the presence of NADPH, but in the presence of an artificial electron donor, cumene hydroperoxide, activity was comparable to that of the FUS enzyme. Further support for a normal structure for the hemoproteins was obtained from type II binding spectra, in which the spectral response was saturated with an equimolar concentration of ketoconazole.**

Cytochrome P-450s comprise a superfamily of heme-thiolate monooxygenases which absorb light at 450 nm upon binding to carbon monoxide in the reduced state and which are widely distributed in eukaryotic and prokaryotic organisms. This superfamily consists of more than 500 genes which are classified into 74 gene families (18). These monooxygenases function as catalysts in the metabolism of numerous exogenous and endogenous compounds, including various drugs, fatty acids, and steroids (19). The cytochrome P-450s in eukaryotes are usually membrane bound and are localized in the endoplasmic reticulum and mitochondria. In contrast, cytochrome P-450s in prokaryotes are water soluble and are localized in the cytoplasm. With few exceptions cytochrome P-450s require NADPH-dependent cytochrome P-450 oxidoreductase (CPR) (in the endoplasmic reticulum) or NA(P)DH-dependent ferridoxin and ferridoxin reductase (in the cytoplasm and in mitochondria) for their catalytic function (17).

Heterologous expression of proteins in *Escherichia coli* has provided an important route for characterizing proteins enzymatically and for biophysical studies. However, in the case of cytochrome P-450 Barnes et al. (3) and Jenkins and Waterman (8) observed expression of CYP17 from the *tac* promoter only after modifying the N terminus of the protein. Low levels of activity in whole cells of *E. coli* expressing bovine 17α-hydroxylase were observed because bacterial cytosolic ferridoxin and ferridoxin reductase supported the cytochrome P-450 activity (8). However, to obtain the full activity of cytochrome P-450 expressed in *E. coli*, purification and reconstitution of activity by adding CPR were required, but these procedures are technically tedious and difficult. Bovine CYP4A1 and human CYP3A4 and CYP17A1 fusions with rat CPR allowed proteins to be produced. These proteins were located in membrane fractions and had catalytic activity, albeit with the N terminus of the P-450s being modified (4, 22, 23).

The *E. coli* system has not been used so far for fungal cytochrome P-450 expression. In *Saccharomyces cerevisiae* sterol 14α-demethylase (CYP51) catalyzes the cytochrome P-450-dependent removal of the 14α-methyl group from lanosterol and is one of the enzymes in the ergosterol biosynthesis pathway. This enzyme is the target for azole antifungal drugs which are used extensively in agriculture and medicine (for a review, see reference 11). Here we describe the expression and activity of the CYP51 and CYP51 fusion (FUS; CYP51 fusion with *S. cerevisiae* CPR) proteins in unmodified form in a study in which we used the *E. coli* expression vector pET15b, which provides a route for convenient purification.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all chemicals used in this study were of analytical grade and were purchased from Sigma Chemicals (Poole, United Kingdom). [32-^{3}H]-3β-hydroxylanost-7-en-32-ol (13.3 mCi/mmol) was a gift from M. Akhtar, Department of Biochemistry, University of Southampton. Restriction endonucleases were obtained from NBL (Northumbria, United Kingdom). Anti-yeast CYP51 immunoglobulin G (IgG) was kindly provided by W.-H. Schunk of Max Delbruck Centre for Molecular Medicine (Berlin, Germany). *E. coli* BL21DE3(pLysS) and pET15b were purchased from Novagen (Madison, Wis.). Ketoconazole was obtained from Janssen Pharmaceuticals (Beerse, Belgium), and organic solvents were obtained from Fisons Chemicals (Loughborough, United Kingdom). Plasmids CYP51:YEp51 and FUS:YEp51 used in this study were constructed in this laboratory (unpublished data).

Recombinant DNA procedures. Recombinant DNA procedures and transformation of *E. coli* cells were performed by following the procedures described elsewhere (21).

Expression and preparation of membrane fractions. BL21DE3(pLysS) strains harboring expression vectors were grown overnight at 37°C and with shaking at 150 rpm in Luria-Bertani (LB) broth containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). Fresh LB broth containing ampicillin and chloramphenicol was inoculated with 1% of the overnight culture, and the organisms were allowed to grow at 37°C and with shaking at 150 rpm. After the cell density had reached optical density at 660 nm of 0.5 to 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added (0.5 mM) and the cultures were allowed to grow for 3 h at 37°C before harvesting at 5,000 × *g* for 10 min. The pellet from 1 liter of

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culture was suspended in 40 ml of buffer A (20 mM Tris-HCl [pH 7.5] buffer containing 0.5 M NaCl), and the suspension was frozen at −70°C overnight. The cells were lysed upon thawing, since strain BL21DE3 carrying pLysS (a plasmid bearing the T7 lysozyme gene) accumulates lysozyme (which induces rapid lysis of cells by freeze-thaw treatment), and sonicated (MSE probe at full power two times for 1 min each time) to shear the genomic DNA present in the lysate. The extract was then centrifuged at 5,000 × *g* for 10 min to remove cell debris, and the resulting supernatant was centrifuged at 100,000 × *g* for 60 min to pellet the membrane fractions. The membrane fractions were suspended in buffer B (100 mM potassium phosphate [pH 7.5] buffer containing 0.1 mM EDTA, 1 mM reduced glutathione, and 20% [vol/vol] glycerol) and were stored at −70°C until further use (21). The protein content in the membrane fractions was measured by using a bicinchoninic acid protein estimation kit (Sigma) with bovine serum albumin standards and by following the instructions provided with the kit.

Western blot (immunoblot) analysis. Whole-cell proteins were fractionated by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis (14) and were then transferred electrophoretically from the gel to a nitrocellulose filter (30). The filter was then probed with rabbit anti-yeast CYP51 IgG, followed by goat antibodies to rabbit immunoglobulin conjugated to alkaline phosphatase (21). Visualization of antibody bound proteins was carried out by following the instructions provided by Boehringer Mannheim in the digoxigenin DNA labelling and detection kit.

Measurement of difference spectra. All spectral data were obtained with a Philips PU8800 UV-visible scanning spectrophotometer. The cytochrome P-450 content in the membrane fractions was determined by measuring the reduced CO difference spectrum, as described by Omura and Sato (20). Type II binding spectra were measured as described by Venkateswarlu et al. (34). The membrane fraction containing 100 pmol of cytochrome P-450 was poured into both sample and reference cuvettes, ketoconazole was added in increments to the sample cuvette after baseline correction, and the difference spectrum from 390 to 500 nm was measured. The CPR content in the membrane fractions was estimated from the rate of cytochrome *c* reduction at room temperature by assuming that 1 nmol of CPR reduces 3 μmol of cytochrome *c* per min (35).

Sterol 14α-demethylation assay. One milligram of membrane protein and 12.2 nmol of [32-^{3}H]-3β-hydroxylanost-7-en-32-ol were added to 0.5 ml of buffer B that had been preincubated at 30°C, and the mixture was shaken at 150 rpm for 20 min with $NADP^+$ (2 mg), glucose phosphate (5 mg), and glucose-6-phosphate dehydrogenase (3 U) to generate NADPH, and then the volume was adjusted to 1 ml with buffer B. After incubating the reaction mixture at 30°C and with shaking at 150 rpm, 0.2-ml aliquots were removed at intervals of 0, 5, 10, 30, and 60 min and were added to 1 ml of a dichloromethane and water (1:1) mixture. The mixtures were immediately vortexed and centrifuged for phase separation. The resulting aqueous phases were washed twice with 0.5 ml of dichloromethane and were treated with charcoal for 1 h at 4°C, and then the radioactivity in the aqueous phases was measured by liquid scintillation counting with a Beckman scintillation counter. For assay mixtures containing cumene hydroperoxide (25 mM), the components of the NADPH-regenerating system and preincubation were omitted (24). The assays were done three times in duplicate to determine the specific rates of activity.

RESULTS

Construction of expression plasmids for *S. cerevisiae* CYP51 and the FUS enzyme. The expression plasmid for CYP51 (CYP51:pET15b) was constructed by subcloning a 1.7-kb fragment containing *S. cerevisiae* CYP51. This was released from plasmid CYP51:YEp51 as a *Sal*I-*Bcl*I fragment and inserted into the *Xho*I and *Bam*HI cloning sites of the pET15b plasmid. The expression plasmid for the fusion construct (FUS:pET15b) was made by using the strategy described below. The FUS: YEp51 yeast expression vector containing the fusion gene was digested with *Xba*I and *Hin*dIII to release a 3.1-kb fragment. The remaining linearized plasmid was digested with *Sal*I to release a 0.4-kb fragment. The 3.1- and 0.4-kb fragments were inserted into the *Xho*I and *Bam*HI cloning sites of pET15b by using a 0.3-kb *Hin*dIII-*Bam*HI fragment, obtained from plasmid YEp51, as a linker. Cloning of the CYP51 and FUS genes into the *Xho*I-*Bam*HI cloning sites of pET15b oriented them in the right direction and in frame and also engineered a six-histidine-residue tail and a thrombin cleavage site at the amino-terminal end of the expressed protein. Each of these plasmids was transformed into *E. coli* BL21DE3(pLysS) to obtain the corresponding recombinant strains.

Expression of CYP51 and FUS proteins in *E. coli*. Figure 1 presents an immunoblot of the cells containing pET15b, CYP51:pET15b, and FUS:pET15b. The bands were detected only in the IPTG-induced cells expressing either the CYP51 or the FUS protein which reacted with anti-CYP51 IgG. The mobilities of the expressed proteins are consistent with their molecular masses deduced from the DNA sequences (55 kDa for CYP51 and 130 kDa for FUS proteins). The immunoblot analysis of the cytosolic and membrane fractions of the induced transformant cells expressing these proteins by probing with anti-CYP51 IgG indicated that the CYP51 and FUS proteins were localized in the membranes (data not shown).

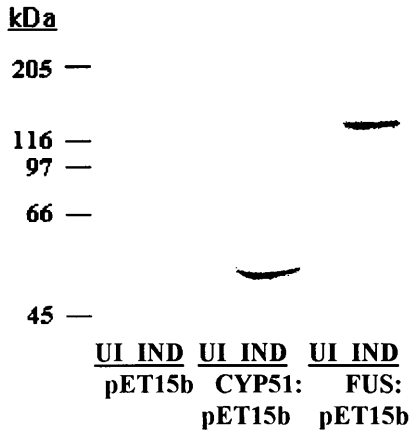

FIG. 1. Western blot of IPTG-induced (IND) and uninduced (UI) cells of the *E. coli* strain transformed with pET15b and the CYP51:pET15b and FUS: pET15b constructs. The contents of whole cells in 100 μl of cell cultures (optical density at 660 nm, 1.0) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to nitrocellulose for reaction with anti-CYP51 IgG.

Cytochrome P-450 and CPR contents in the membrane fractions of the recombinant *E. coli* cells. Only the membrane fractions of the transformed *E. coli* strains expressing the CYP51 and FUS enzymes produced CO-reduced difference spectra with a Soret peak at 448 nm (Fig. 2). The cytochrome P-450 and CPR contents in the membranes of the *E. coli* strains expressing CYP51 and FUS proteins were determined and are presented in Table 1. The specific content of cytochrome P-450 in CYP51 membrane fractions was approximately two-thirds of that in FUS membrane fractions. As expected, no reductase (on the basis of cytochrome *c* reducing activity) was detected in CYP51 membranes. The ratio of cytochrome P-450 and CPR in FUS membranes was estimated to be about 1:0.9 (mol/mol).

Sterol 14α-demethylation catalyzed by CYP51 and FUS enzymes. The activities of heterologously expressed CYP51 and the FUS enzyme were tested. Membrane fractions isolated from the recombinant *E. coli* strains expressing these proteins were incubated with the radiolabelled substrate [32-^{3}H]-3β-hydroxylanost-7-en-32-ol, and the radiolabelled formic acid produced by the 14α-demethylation reaction was measured. The catalytic activities of CYP51 and the FUS enzyme in the presence of the NADPH-regenerating system or cumene hydroperoxide are presented in Fig. 3. The membrane fraction containing the FUS enzyme catalyzed the 14α-demethylation reaction in the presence of NADPH (Fig. 3A). In contrast, the NADPH-dependent activity was not observed in the CYP51 membrane fraction. This result is expected because of the absence of endogenous CPR, which is necessary for cytochrome P-450 to accept electrons from NADPH for the oxidation reaction, in this membrane fraction. In the presence of an artificial electron donor, cumene hydroperoxide, both CYP51 and FUS membrane fractions were active in the 14α-

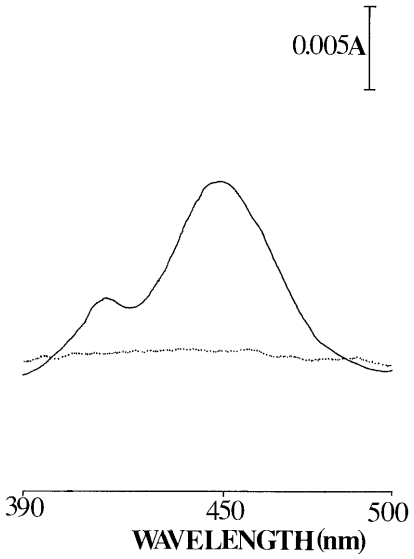

FIG. 2. A typical CO difference binding spectrum obtained with the microsomal fractions containing CYP51 (dotted line, baseline).

demethylation of the substrate, since the transfer of electrons from cumene hydroperoxide to the cytochrome P-450s does not require CPR (Fig. 3B). However, the activity observed in the presence of cumene hydroperoxide was only about one-fourth of that observed in the presence of the NADPH-regenerating system, and since the activity did not change even after increasing the concentration of cumene hydroperoxide in the assay (data not shown), it is suggested that this cytochrome P-450 may not be very efficient in accepting electrons from artificial electron donors. The 14α-demethylation reaction was linear with time for at least 60 min. The specific rates of the demethylation reaction of the CYP51 and FUS enzymes in presence cumene hydroperoxide were 0.46 ± 0.11 and 0.45 ± 0.15 nmol/min/nmol of P-450, respectively, and that of the FUS enzyme in presence of NADPH was 1.96 ± 0.37 nmol/min/nmol of P-450.

Azole drug binding. The addition of ketoconazole, an azole drug, to the CYP51 membrane fraction induced a type II binding spectrum with a maximum absorbance at 428 nm and a minimum at 409 nm (Fig. 4), indicative of a shift in the heme iron of the cytochrome P-450 to low-spin state upon inhibitor binding (7). A similar spectrum was also obtained by adding ketoconazole to the FUS membrane fraction. The magnitude of the spectral response increased steadily by increasing the ketoconazole concentration and attained saturation when an equimolar concentration of ketoconazole was added to the cytochrome P-450 of the CYP51 and FUS membrane fractions (Fig. 5). The half-saturation concentration of ketoconazole was calculated to be 0.25 μM for both CYP51 and FUS enzymes. These data suggest that both CYP51 and its fused enzyme, FUS, exhibited a similar affinity for ketoconazole.

TABLE 1. Cytochrome P-450 and CPR contents in the membrane fractions and yields of the transformed *E. coli* strains[a]

Plasmid	Cytochrome P-450		CPR	
	pmol/mg of protein	nmol/liter	pmol/mg of protein	nmol/liter
CYP51:pET15b	12.8 ± 2.6	1.4 ± 0.3	ND[b]	ND
FUS:pET15b	17.4 ± 3.7	2.0 ± 0.4	15.7 ± 1.1	1.8 ± 0.5

[a] Specific contents are means ± standard deviations of three experiments.
[b] ND, not detected.

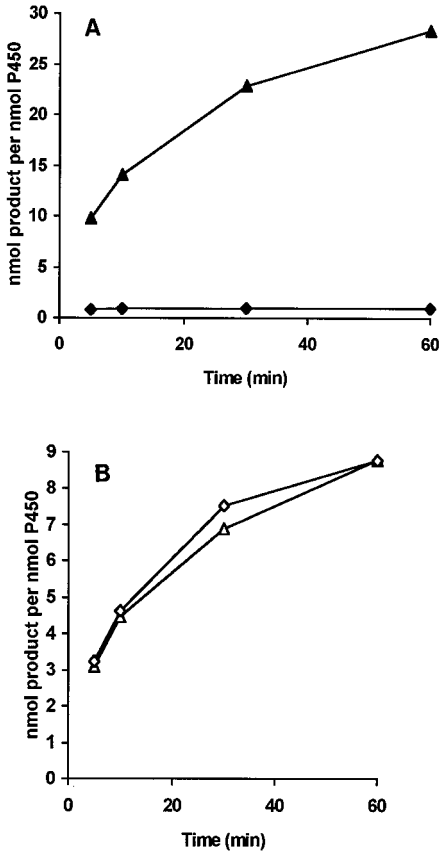

FIG. 3. 14α-Demethylation of substrate by the membrane fractions expressing CYP51 and FUS enzymes in presence of the NADPH-regenerating system (A) and cumene hydroperoxide (B). The assays for each time point were done in duplicate, and the average is presented. (A) ▲, FUS:pET15b; ◆, CYP51:pET15b. (B) △, FUS:pET15b; ◇, CYP51:pET15b.

DISCUSSION

S. cerevisiae CYP51 is one of the few fungal cytochrome P-450s which have been extensively studied (16). The CYP51s of several species, including *S. cerevisiae*, *Candida albicans*, rats, pigs, and humans, have been purified and characterized (2, 6, 26, 27, 31, 39) and have also been cloned from many of these species (1, 9, 10, 13, 15, 25, 28, 33). In addition, plant CYP51 was recently studied by using microsomes obtained from maize embryos (29) and cloned from wheat (3a). CYP51 is the only cytochrome P-450 known so far to be present in fungi, plants, and mammals, in which it is involved in the biosynthesis of ergosterol, sitosterol, and cholesterol, respectively. Its presence in organisms belonging to different phyla suggests that ancestral forms of this enzyme were present early in evolution (18). However, the substrate of CYP51 is not the same in all these organisms, i.e., lanosterol, eburicol, dihydrolanosterol, and obtusifoliol are the substrates in yeast, filamentous fungi, animals, and plants, respectively (38). In addition, azole antifungal drugs, which have been used successfully for treating plant as well as animal fungal infections, have a higher affinity for CYP51 in fungi than for the CYP51 in plants and animals (32). The selective inhibition of fungal CYP51 by

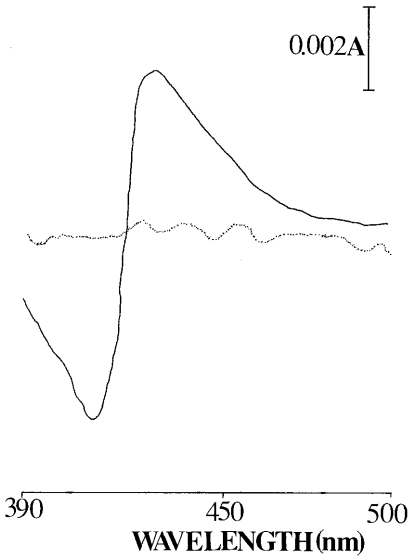

FIG. 4. Type II binding spectrum obtained by adding an equimolar concentration of ketoconazole (100 pmol) to CYP51 (100 pmol)-containing membrane fraction (dotted line, baseline).

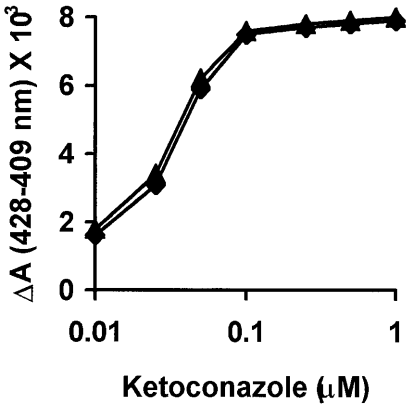

FIG. 5. Saturation curves of type II binding spectra of CYP51 and FUS enzymes obtained by adding ketoconazole in increments to 100-pmol equivalents of cytochrome P-450 of membrane fractions expressing CYP51 and FUS enzymes. ΔA, difference between maximum absorbance at 428 nm and minimum absorbance at 409 nm. The binding studies were carried out in duplicate, and the average changes in absorbance observed with each concentration of keconazole were calculated for this figure. ◆, CYP51:pET15b; ▲, FUS:pET15b.

azoles is not understood very well at the molecular level due to the difficulties associated with membrane-bound protein structural (three-dimensional structure) elucidation by X-ray crystallography. Large amounts of highly purified protein are needed for this and other biophysical techniques, hence our interest in comparing methods of heterologous expression.

Many eukaryotic cytochrome P-450s (particularly mammalian P-450s), which are otherwise expressed very poorly, were expressed at very high levels in *E. coli* by changing their N termini (for reviews, see references 5 and 36). However, CYP51 and FUS enzymes were expressed successfully in *E. coli* without the N-terminal modification, were located in the membrane, and were active in catalyzing the 14α-demethylation reaction. No difference in the catalytic activities of these two proteins was observed in the presence of an artificial electron donor, cumene hydroperoxide. However, the catalytic activity observed with the FUS enzyme in the presence of the NADPH-regenerating system is higher than that found in the cumene hydroperoxide-containing assays. This result is similar to those observed by Wiseman and Woods (37) and King et al. (12) in benzo(*a*)pyrene hydroxylation by yeast microsomal cytochrome P-450 or by purified cytochrome P-450, and this observation stresses the need for CPR for achieving higher catalytic activities of cytochrome P-450s. The expressed FUS enzyme metabolized 3β-hydroxylanost-7-en-32-ol with a turnover number of 1.96 ± 0.37 min^{-1}. This is comparable to the turnover numbers reported for CYP51 purified from rat (0.11 to 0.38 min^{-1}) (31), pig (1.35 min^{-1}) (26), and human (0.17 min^{-1}) (27) liver microsomes and for detergent-solubilized membrane proteins of *E. coli* expressing human CYP51 (0.97 min^{-1}) (28). However, the turnover number was low compared to that of the CYP51 purified from *S. cerevisiae* microsomes (5.0 min^{-1}) (38), although the ratio of cytochrome P-450 and CPR in the FUS enzyme was quite close to that found in the assay with reconstituted enzyme. The variation in catalytic activity between *E. coli*-expressed FUS enzyme and purified CYP51 is probably due to differences in the assay conditions used, and this view is supported by the type II binding studies. The CYP51 and FUS proteins expressed in the transformed *E. coli* membrane fractions bound ketoconazole, as indicated by the type II binding spectrum, and showed a 1:1 stoichiometry in interacting with the drug, as was previously reported for the purified CYP51 protein (40). This result also indicates that the affinity of CYP51 was not affected by its fusion with CPR. The expression of catalytically active *S. cerevisiae* CYP51 fused with CPR in *E. coli* in this study suggests the possibility that this system can be used to screen for CYP51 antifungal inhibitors and that other catalytically self-sufficient fungal cytochrome P-450s which are industrially important in *E. coli* can be expressed by fusion with CPR. The overall yield of CYP51 and FUS might be improved by use of the *tac* promoter and by modifying the N terminus as undertaken for human CYP51 (28). In that study up to 240 nmol/liter was obtained (cf. the approximately 2 nmol/l for CYP51 and FUS obtained here), but we show that authentic yeast CYP51 can be produced conveniently with the capacity for simple purification by using the N-terminal histidine tag. Furthermore, a catalytically self-sufficient fusion protein can be produced in a similar way, which may be of assistance in studies of the structure of this protein-protein interaction.

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