Generation of a Complete, Soluble, and Catalytically Active Sterol 14α-Demethylase–Reductase Complex †

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Generation of a Complete, Soluble, and Catalytically Active Sterol 14α-Demethylase−Reductase Complex[†]

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ABSTRACT: Sterol 14α-demethylation is one of the key steps of sterol biosynthesis in eukaryotes and is catalyzed by cytochrome P450 sterol 14α-demethylase (other names being CYP51 and $P450_{14DM}$) encoded by *ERG11*. This enzyme activity is supported by an associated NAPDH-dependent reductase encoded by *NCPR1* (*NCP1*), which is also associated with the endoplasmic reticulum. A diglycine linker recognition site (Gly-Gly-Ile-Glu-Gly-Arg-Gly-Gly) for the protease factor Xa, also containing a thrombin recognition site, was inserted just beyond the N-terminal hydrophobic segment of *Candida albicans* Erg11p. This modified enzyme was heterologously expressed at a level of 2.5 nmol of Erg11p/mg of protein as an integral endoplasmic reticulum protein. Following purification, treatment of the modified protein with factor Xa or thrombin resulted in sequence-specific cleavage and production of a soluble N-terminal truncated Erg11p which exhibited spectral characteristics identical to those of the purified full-length, wild-type form. Furthermore, reconstitution of the soluble enzyme with soluble yeast Ncpr1p, expressed and purified as an N-terminal deletion of 33 amino acids encompassing its membrane anchor, resulted in a fully functional and soluble eukaryotic Erg11p system. The complex was disrupted by high-salt concentration, reflecting the importance of electrostatic forces in the protein−protein interaction. The results demonstrate the membrane anchor serves to localize Erg11p to the ER where the substrate is located, but is not essential in either Ncpr1p or Erg11p activity. The possibility of cocrystallization of an active soluble eukaryotic 14α-demethylase can be envisaged.

Sterol biosynthesis is ubiquitous in eukaryotes, and the membrane-bound nature of most of the enzymes involved presumably reflects the hydrophobic nature of the substrates that are dissolved in the membranes. The molecular basis of the membrane associations for these enzymes is an area of interest for determining whether this is needed for activity or protein−protein interactions between the various biosynthetic enzymes and their electron transport partners. Generating soluble enzymes may also facilitate crystallization of the membrane-bound components of the sterol biosynthetic apparatus, leading to fundamental information and greater understanding of anti-sterol inhibitors for medicine and agriculture.

We have been interested in studying the microsomal Erg11p (other names being CYP51 and $P450_{14DM}$) from *Candida albicans* and have been probing both the mechanism of 14α-demethylation (*1*) and predicted structural features of this enzyme (*2*). Erg11p has been identified as the only cytochrome P450 form found in animals, plants, and fungi as well as in *Mycobacterium tuberculosis*, suggesting that it represents an ancient metabolic activity within the superfamily of P450 enzymes (*3*). Inhibition of fungal Erg11p is also of applied interest as azole antifungals are central to therapy of mycoses in the clinic (*4*).

Membrane associations of proteins can be investigated using a range of approaches. These can include removal of the hydrophobic membrane anchor. For cytochromes P450, approaches have included insertion of a fXa protease recognition site (*5*), expression of N-terminal truncated forms of proteins (*6*), or introduction of a thrombin sensitive site beyond the membrane anchor and subsequent generation of a soluble derivative after protease digestion (*7*). In the investigation of membrane-bound enzymes of sterol biosynthesis, generation of soluble forms of such proteins has been restricted to date to trypsin proteolysis such as for squalene synthetase (*8*). In this paper, we demonstrate the production of a soluble and enzymatically active *C. albicans* Erg11p, which required the introduction of a Xa protease site flanked by two glycines on either side. This soluble eukaryotic Erg11p can couple and receive electrons from its soluble redox partner, Ncpr1p, the first such demonstration of a cytochrome P450 activity or sterol biosynthetic protein−protein interaction. The implications for the importance of membrane association in sterol biosynthetic enzymes are discussed.

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MATERIALS AND METHODS

Recombinant DNA Manipulations. The expression vector containing the *C. albicans ERG11* gene inserted into the yeast expression plasmid YEp51 was described previously (*1*) and used as a template for PCR mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers: outside primer 1 (5′-CGCGTCGACATAATGGCTATTGTTGAAACTGTC-3′) annealing to positions 1−21 of the open reading frame and containing a 5′ added *Sal*I site, inside primer 1 (5′-TCTGCAGCTGGTGGTATCGAAGGTAGAGGTGGTCAACCT-3′) containing the factor Xa protease recognition site Ile-Glu-Gly-Arg flanked on either side by two Gly residues and creating a thrombin recognition site Arg-Gly, inside primer 2 (5′-AGGTTGACCACCACCTCTACCTTCGATACCACCAGCTGCAGA-3′) overlapping with inside primer 1, and outside primer 2 (5′-TGGCATATGCATTCTGAGAGTTTCCTT-3′) annealing to the 3′ end of the open reading frame and containing the restriction site *Bgl*II. In the first PCR steps, two separate reactions were performed using outside primer 1′ and inside primer 2, and inside primer 1 and outside primer 2. The overlapping DNA fragments were purified, mixed, and recombined in a final PCR step using outside primer 1 and outside primer 2. The resulting Erg11p(fXa-link) was cloned in a ligation reaction involving the *Sal*I−*Bgl*II-cut PCR fragment and the *Sal*I−*Bgl*II-cut *C. albicans* Erg11p expression plasmid, YEp51:Erg11p. Full-length yeast Ncpr1p and the N-terminal truncated form, Ncpr1p [33], were isolated by PCR using pFBY4 containing yeast Ncpr1p as a template and subsequently cloned into YEp51 as described previously (*9*). All PCRs were carried out using Pfu polymerase (Stratagene) and a Perkin-Elmer DNA thermal cycler. Transformants were screened by restriction digestion and confirmed by sequencing. The desired Erg11p(fXa-link) was transformed into the yeast strain AH22 (MATa, *leu 2-3, 2-112, his 4-519*), and the *NCPR1* clones were transformed into yeast strain JL20 (MAT a, *leu2-3, 2-112, his4-519, ade1-100, ura3-52*).

Expression and Purification of Recombinant Erg11p Proteins. Yeast transformants were cultivated in yeast minimal medium containing 1.34% (w/v) Difco yeast nitrogen base without amino acids, 20 μg/mL L-histidine, and 2% (w/v) glucose as the 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 induction for 24 h, cells were harvested by centrifugation and mechanically disrupted (*10*). Microsomes were obtained following 100000*g* ultracentrifugation, and the expressed Erg11p was solubilized with 2% (w/v) sodium cholate and purified on ω-amino-*n*-octyl Sepharose 4B and hydroxyapatite as previously described (*2*).

Expression and Purification of Recombinant Ncpr1p Proteins. JL20 transformants carrying wild-type and truncated yeast Ncpr1p expression vectors were grown in yeast minimal medium containing Difco yeast nitrogen base without amino acids (1.34%, w/v), 20 μg/mL 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 (*1*). Cells were harvested by centrifugation and subjected to mechanical breakage. Microsomal and cytosolic components, containing full-length and soluble Ncpr1p, were isolated by ultracentrifugation. Purification of wild-type Ncpr1p was carried out according to published procedures (*11*). Soluble Ncpr1p was purified by hydroxyapatite and 2′,5′-adenosine diphosphate agarose (2′,5′-ADP-agarose) chromatography as described previously (*9*).

Proteolysis with fXa and Thrombin Protease. Purified Erg11p fractions were dialyzed against protease cleavage buffer [100 mM NaCl/50 mM Tris-HCl (pH 8.0)/1 mM $CaCl_2$ containing 20% (v/v) glycerol]. Site-specific cleavage was performed in the same buffer system containing 1% (v/v) Triton X-100. Lyophilized fXa (Boehringer Mannheim) was freshly reconstituted in distilled water and added to the cleavage reaction mix to give a final Erg11p:fXa ratio of 5:1. In our reactions, 10 nmol of highly purified Erg11p (0.5 mg) was treated with 0.1 mg of fXa. Digestions were performed overnight at 4 °C. In a typical cleavage reaction with thrombin, Erg11p(fXa-link) was treated with a molar ratio of 50:1 for 30 min at 37 °C in the presence of 1% (v/v) Triton X-100.

Purification of Shortened Erg11p(fXa-link). After proteolysis with fXa, the cleaved Erg11p was purified from uncleaved enzyme employing *n*-octyl Sepharose 4B which had been equilibrated with 500 mM potassium phosphate buffer (pH 7.2) containing 20% (v/v) glycerol, 0.5 mM EDTA, and 1.0 mM DTT. The column was washed with equilibration buffer, thus eluting the cleaved protein. The uncleaved protein was eluted with the same buffer but in the presence of 0.5% (v/v) Tween 20. Subsequently, protease digestion was monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Reconstitution of CYP51 Activity with Full-Length and Soluble Ncpr1p. Each reaction mixture contained either wild-type or soluble purified Erg11p (0.2 nmol) and 1 unit of purified wild-type or soluble yeast Ncpr1p in a total volume of 50 μL. One unit converts 1 μmol of cytochrome *c* to its reduced form per minute. For reconstitution studies, 13 nmol of substrate, 24-methylene-24,25-dihydrolanosterol, was dispersed in 50 μg of 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 reaction mixtures were incubated at 37 °C for 20 min and were terminated by the addition of 5 mL of methanol. Sterol substrate and metabolite were extracted and analyzed by gas chromatography−mass spectroscopy (*12*).

Spectrophotometric Measurements. A Philips PU8800 UV/VIS scanning spectrophotometer was used for all spectral studies. The cytochrome P450 concentration was estimated according to ref *13*. Spectral studies of fluconazole binding were performed on purified wild-type and soluble *C. albicans* Erg11p. Fluconazole, dissolved in DMSO, was added direct to the sample cuvette; the contents were mixed, and after 1 min, the spectrum between 500 and 350 nm was recorded. Via the addition of several increments of fluconazole, the change in absorbance between the type II peak (420−427 nm) and the corresponding trough (390−410 nm) was related to the amount of added azole antifungal. The maximum concentration of DMSO that was used [1% (v/v)] caused no

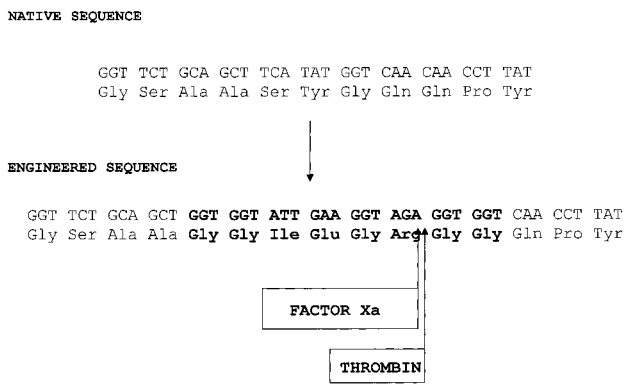

FIGURE 1: Amino terminal sequence of *C. albicans* Erg11p and its modification creating a factor Xa protease recognition site flanked by two glycine residues on either side (boldface). The wild-type sequence was modified by PCR site-directed mutagenesis to produce the tetrapeptide recognition sequence for factor Xa, Ile-Glu-Gly-Arg. The insertion of the glycine residues, two on either side of the factor Xa recognition site, allowed accessibility of factor Xa to the protease site while also creating a thrombin protease recognition site, Arg-Gly.

change in the spectrum over the region that was scanned. Cytochrome *c* reductase activity was measured as described previously (*14*). The absorption spectrum of the purified yeast Ncpr1p [Δ33] was obtained by scanning from 300 to 700 nm (*15*).

RESULTS

Construction of Protease fXa and Thrombin Recognition Sites. The six-amino acid sequence, Gly-Gly-Ile-Glu-Gly-Arg-Gly-Gly, containing both the fXa protease recognition sequence Ile-Glu-Gly-Arg and the thrombin recognition site Arg-Gly was engineered into the *C. albicans* Erg11p at positions 63−66, replacing the wild-type sequence, Ser-Tyr-Gly-Gln, with the insertion of four extra glycine residues into the protein, two on either side of the fXa protease recognition site (Figure 1).

Expression of Wild-Type and Mutant Erg11p and Ncpr1. No difference was observed in the expression levels of the wild-type protein and Erg11p(fXa-link). The specific heme content exceeded 2.5 nmol/mg of microsomal protein after expression from the GAL10 promoter of YEp51 when determined by the optical absorption spectrum of the carbon monoxide-bound form of the reduced enzyme. The expression system had already included modifying the coding sequence at position 263 due to the presence of CTG, which encodes leucine in *Saccharomyces cerevisiae*, but serine in *C. albicans* (*16*). Introduction of TCT allowed the authentic amino acid (serine) to be included when expressed in *S. cerevisiae*. The absorption maxima of the carbon monoxide-bound forms of both Erg11p and Erg11p(fXa-link) were located at 448 nm with no peaks at 420 nm (P420 is an inactive form of the protein), indicating stable production of the mutant and wild-type enzymes. Wild-type and soluble yeast Ncpr1p localized in the microsomal and cytosolic fractions of *S. cerevisiae*, respectively, after expression from the GAL10 promoter of YEp51 (*9*). The yeast Ncpr1p contents were determined using the cytochrome *c* reduction assay.

Purification of Erg11p(fXa-link) and Soluble Ncpr1p. Microsomal wild-type and mutated Erg11p were solubilized with sodium cholate and purified by ω-amino-*n*-octyl Sepharose 4B chromatography as previously described (*2*). Each purified protein preparation was homogeneous as assayed by SDS−PAGE and specific heme content, which was greater than 17 nmol/mg of protein. Microsomal wild-type yeast Ncpr1p was purified as previously described (*11*). Heterologously expressed cytosolic-located soluble yeast Ncpr1p was precipitated between 40 and 65% (w/v) ammonium sulfate and purified following hydroxyapatite and 2′,5′-ADP-agarose chromatography (*9*). The purified yeast Ncpr1p [33] reduced cytochrome *c* at a rate of 24.3 μmol min^{-1} (mg of protein)$^{-1}$ and was homogeneous as determined by SDS−PAGE with an apparent molecular mass of 75 000 Da compared with 78 000 Da for the wild-type Ncpr1p.

Cleavage Conditions for Erg11p. Experiments with *C. albicans* Erg11p following mutation of the sequence to include a single fXa site located at positions 63−66 in the protein failed to allow cleavage following treatment with fXa, revealing the inaccessibility of this site to the protease (unpublished observations). Site-directed mutagenesis to allow placement of the fXa protease site, instead of wild-type amino acids, at positions 34−37, 40−43, 59−62, and 68−71, which are also after the putative hydrophobic membrane anchor (positions 13−33), led only to the heterologous production of inactive enzyme (cytochrome P420). Similarly, inactive enzyme was produced in attempts to express N-terminal truncated forms lacking 40 and 62 amino acids. Consequently, these constructs were not used further. In an attempt to make the fXa protease site more accessible at positions 63−66, two glycine residues were inserted on either side of the recognition site by genetic engineering. To ensure maximal accessibility and efficiency of cleavage, proteolysis was carried out with purified Erg11p in the presence of detergent [1% (v/v) Triton X-100, final concentration]. The major cleavage product migrated during SDS−PAGE with an apparent molecular mass of 45 000 Da, compared to 55 000 Da for purified wild-type Erg11p. Proteolysis with thrombin also liberated a 45 000 Da product corresponding to the cleaved Erg11p, as insertion of the glycines created an Arg-Gly thrombin site adjacent to the factor Xa site. No cleavage products were observed following treatment of wild-type Erg11p with either fXa or thrombin as expected from the amino acid sequence of the protein.

Purification and Spectral Properties of the Cleaved, Soluble Erg11p. The cleaved, soluble Erg11p protein was separated from the uncleaved protein by hydrophobic chromatography employing *n*-octyl Sepharose 4B. Uncut, full-length Erg11p bound to the chromatograhic material through interaction with its hydrophobic membrane anchor, whereas the cleaved protein passed unbound through the column. The purified truncated protein was homogeneous as determined by SDS−PAGE with an apparent molecular mass of 45 000 Da, corresponding to the loss of the 10 000 Da amino terminus encompassing the proposed membrane anchor (Figure 2). Purified truncated and wild-type Erg11p both exhibited identical spectral properties with the carbon monoxide-reduced absorbance maximum located at 448 nm (Figure 3A). Fluconazole, the most widely used clinical azole antifungal, bound to the heme of the truncated protein as a sixth ligand producing the typical type II spectrum with a spectral maximum located at 424 nm and a spectral minimum at 409 nm (Figure 3B). Identical spectra were produced for

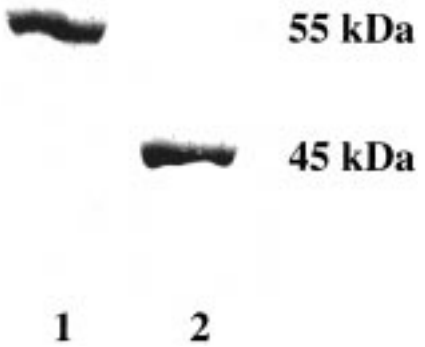

FIGURE 2: Purification of the cytosolic domain of *C. albicans* Erg11p. Purified proteins (2 μg) were analyzed on a 10% polyacrylamide−SDS gel and visualized with Coomassie Blue: lane 1, purified, wild-type Erg11p; and lane 2, purified, soluble catalytic domain of Erg11p following cleavage of Erg11p(fXa-link) with factor Xa and purification on *n*-octyl Sepharose.

fluconazole interaction with wild-type and uncleaved Erg11p with saturation occurring at equimolar amounts (Figure 4). This revealed no major effect on the heme environment or gross conformational changes at the active site following fXa cleavage. This titration, with saturation at equimolar amounts, has been observed between *C. albicans* Erg11p and fluconazole previously by us (*2*) and others (*17*), and the IC_{50} for saturation corresponded to half the amount of fluconazole as reported previously.

Reconstitution of fXa-Cleaved, Soluble Erg11p with Soluble Yeast Ncpr1p. Soluble Erg11p could catalyze sterol 14-demethylation with activity similar to that of the wild-type protein. This was shown in complete reconstituted systems containing full-length, wild-type yeast Ncpr1p where soluble Erg11p could catalyze the 14α-demethylation of 24-methylene-24,25-dihydrolanosterol. Turnover numbers were comparable to those for wild-type Erg11p (Table 1). Furthermore, purified, soluble Erg11p could interact with soluble yeast Ncpr1p and catalyze sterol 14α-demethylation (Table 1), again with activities similar to those following reconstitution of wild-type Erg11p with wild-type yeast Ncpr1p (*1*, *2*, *9*, *17*). In most experiments, values between 0.2 and 0.3 nmol of product min^{-1} (nmol of Erg11p)$^{-1}$ were obtained. Within-experiment variation in enzyme turnover was observed to be around 20%, but lower activities were observed in occasional reactions, which we ascribe to subtle changes in the conditions of reconstituting the proteins with insoluble substrate. As we noted previously, soluble Ncpr1p could support the activity of wild-type *C. albicans* Erg11p (*9*).

Reconstitution of fXa-Cleaved, Soluble Erg11p with Soluble Yeast Ncpr1p in the Presence of Salt. The ability of soluble yeast Ncpr1p to bind soluble Erg11p in an ionic strength-dependent manner strongly suggests that electrostatic forces are pivotal in this interaction. As shown in Figure 5, soluble Erg11p activity, as reconstituted with soluble Ncpr1p, is inhibited at high ionic strengths (>200 mM NaCl). Consequently, soluble yeast Ncpr1p is essentially unable to provide electrons to soluble Erg11p above 200 mM NaCl. Comparison of the effect of ionic strength on activity supported by wild-type Erg11p and Ncpr1p revealed relative resistance to repulsion when compared to their soluble counterparts so that sterol 14α-demethylation catalyzed by the wild-type enzymatic complex occurred at ionic strengths which abolished activity in reconstitutions of soluble Erg11p with soluble Ncpr1p. The hydrophobic N-termini stabilized the complex under these conditions, although it was not required for activity in the absence of salt.

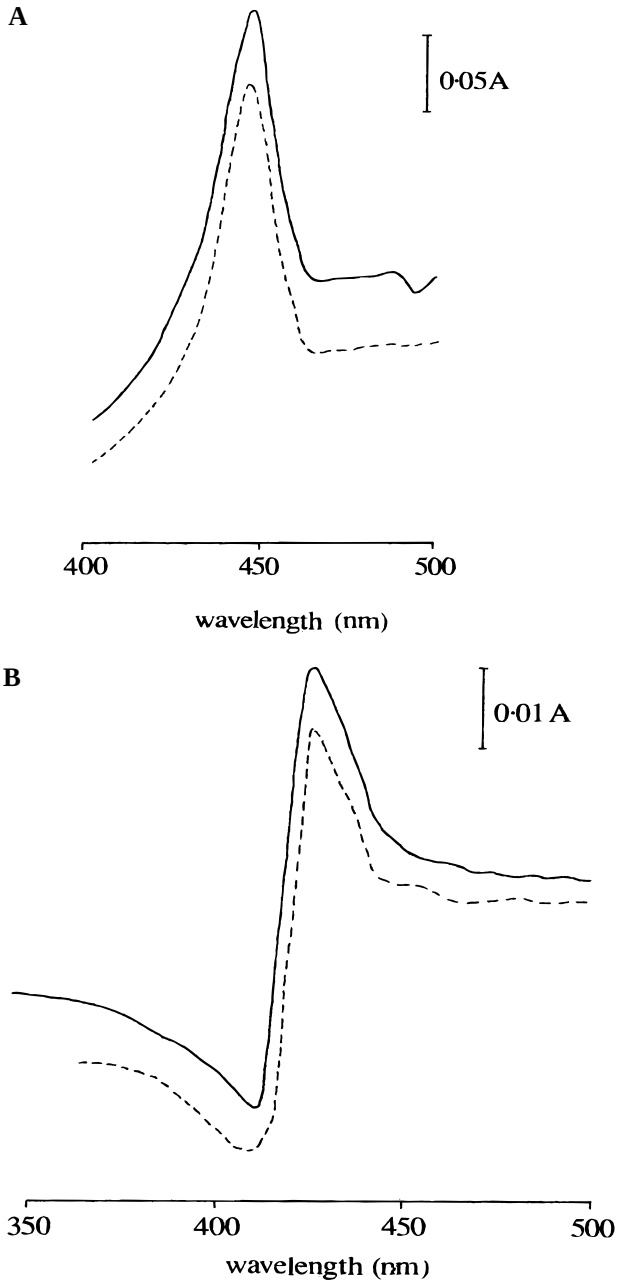

FIGURE 3: (A) Comparison of the reduced carbon monoxide difference of purified wild-type and soluble *C. albicans* Erg11p. The reduced carbon monoxide difference spectra of purified wild type (—) and soluble (- - -) Erg11p, prepared as described in Materials and Methods. (B) Type II binding spectra of wild-type and soluble *C. albicans* Erg11p induced by fluconazole. This interaction is characterized by the displacement of the native sixth ligand of the Erg11p heme iron (water molecule) by the nitrogen atom in the triazole ring of fluconazole and resulted in a spectral peak located at 424 nm and a trough located at 409 nm. The type II spectra of purified wild-type (—) and soluble (- - -) Erg11p were prepared as described in Materials and Methods.

DISCUSSION

Predictions of membrane-binding domains of the post-squalene enzymes of sterol biosynthesis indicate N-terminal anchors are common as has been shown here for sterol 14α-demethylase and previously for Ncpr1p (*9*). Anchoring of

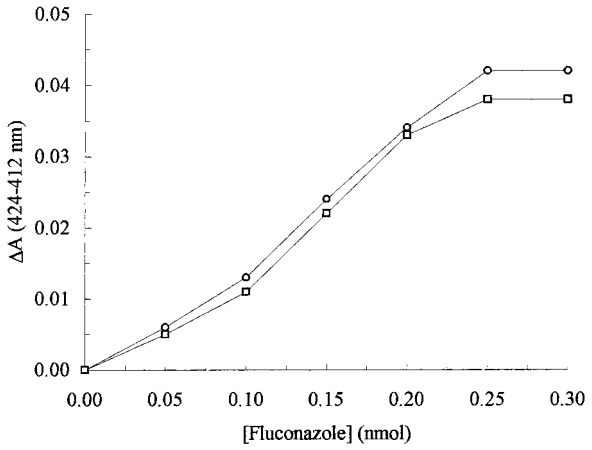

FIGURE 4: Spectral titration of purified, ferric wild-type (○) and soluble (□) *C. albicans* Erg11p fluconazole. Wild-type and soluble Erg11p was diluted with potassium phosphate buffer (pH 7.2) and titrated with increasing amounts of fluconazole. The magnitude of the resulting difference spectrum was plotted as a function of fluconazole amount. The results are from a representative experiment of three; values of ΔA varied by <5% between experiments.

Table 1: Sterol 14α-Demethylase Activity of Purified Wild-Type and Soluble *C. albicans* Erg11p in a Reconstituted System Containing Purified Wild-Type and Soluble Ncpr1p[a]

	sterol 14α-demethylase activity[b]	
system	wild-type Erg11p	soluble Erg11p
purified, wild-type yeast Ncpr1p	0.24 ± 0.04	0.20 ± 0.03
purified, soluble yeast Ncpr1p	0.22 ± 0.03	0.18 ± 0.03

[a] Each reaction mixture contained either 0.2 nmol of purified wild-type or soluble Erg11p and 1 unit of purified wild-type or soluble yeast Ncpr1p and 50 μg of dilauroylphosphatidylcholine (DLPC). The ratio of Erg11p:Ncpr1p was 1:30. No activity was detected in the absence of either Erg11p or the reductase component with the presence of NADPH being essential. Experiments were performed in triplicate, and results are shown ± standard deviation. [b] Activity expressed as nanomoles of product formed per minute per nanomole of Erg11p.

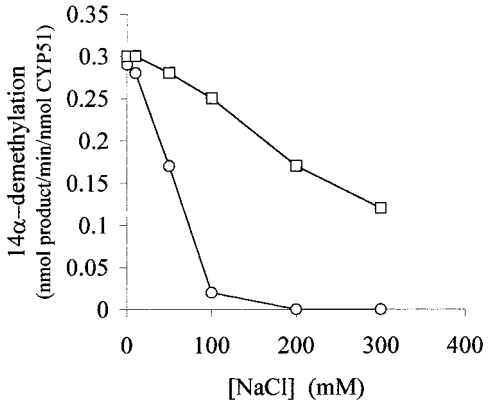

FIGURE 5: Effect of high salt concentrations on reconstituted Erg11p. Wild-type Ncpr1p (□) and soluble Erg11p and soluble Ncpr1p (○) were reconstituted in purified systems, according to Materials and Methods, and sterol 14α-demethylase activity was measured in the presence of varying concentrations of sodium chloride. Results are the means of triplicate experiments with sterol 14α-demethylase activity.

the protein to the membrane probably locates the catalytic domain of the enzyme at a site where access to their substrates is maximal, as the substrates are highly hydrophobic with high partition coefficients.

The results of this study are in accord with the idea that membrane attachment is a result of insertion of a single membrane-spanning anchor for *C. albicans* Erg11p. Following genetic engineering to introduce a factor Xa site at positions 63−66, no effective cleavage was produced with the membrane-bound or purified enzyme, indicating the enzyme structure blocked access to the protease site. Movement of the site was undertaken, but only inactive protein was observed from constructs after heterologous expression. Similarly, heterologous expression of an enzyme lacking the first 62 amino acids and of the first 40 failed to produce detectable P450 spectra, indicating incorrect conformation of the protein that was produced. We assume these mutant proteins, which exhibited a reduced carbon monoxide difference spectrum with a Soret maximum at 420 nm indicative of an inactive protein, arose from incorrect folding. Further, exhaustive approaches were not made, and the possibility that different mutations or experimental approaches might prove to be successful cannot be ruled out. The observation of a correctly folded enzyme after addition of a fXa site at residues 63−66, but which could not be cleaved, prompted us to attempt addition of a diglycine linker to either side of the fXa site. This was undertaken to facilitate the accessibility of the protease site and proved to be successful. A normal reduced carbon monoxide difference spectrum was observed for the mutant protein, which now also contained a thrombin site. Protein cleavage with factor Xa and thrombin protease released a soluble and catalytically active truncated Erg11p. This relative position was likely to have been successful for this protease site insertion due to it being located between the membrane anchor and the globular domain of the Erg11p. The spectral characteristics observed on binding fluconazole to soluble Erg11p were identical to those of full-length Erg11p, indicating removal of the hydrophobic N-terminus plays no role in azole drug binding. The results for both Erg11p proteins were as observed in previous studies for binding of this major antifungal drug to the target Erg11p of *C. albicans* (*2*, *17*).

It has been proposed that the major role of the N-terminal hydrophobic tail of Erg11p was to provide a membrane anchor and facilitate interaction with the membrane phospholipid associated with 14α-methyl intermediates of sterol biosynthesis (*18*). Other charged residues may also be important for interaction between the membrane and the substrate access channel (*19*). However, the results here support the concept that the N-terminus serves only as a membrane anchor. The coupling observed between soluble Erg11p and soluble Ncpr1p shows the NH_2-terminal portion of both proteins plays no essential role in either interaction of fungal Erg11p and its associated reductase, Ncpr1p, or electron transfer from NADPH via Ncpr1p to the soluble Erg11p for catalyzing 14α-demethylation. Soluble yeast Ncpr1p couples with soluble Erg11p, but is disrupted by high salt concentrations, indicating electron transfer results solely from the electrostatic interaction between the protein components of the system.

In contrast to yeast Ncpr1p, mammalian reductase requires the hydrophobic N-terminus of the protein for coupling to mammalian cytochrome P450 (*9*, *20*). Previous studies involving molecular modeling of eukaryotic P450s (based on the resolved crystal structures of the prokaryotic soluble P450s $P450_{cam}$, $P450_{terp}$, and $P450_{BM3}$) suggested interaction

of the membrane-spanning segments of both P450 and Ncpr1p occurred, allowing P450/Ncpr1p recognition and electron transfer (*21*), but this is not supported for the sterol biosynthetic yeast enzymes studies here.

The results presented here build on previous observations that soluble yeast Ncpr1p is catalytically active in contrast to the mammalian homologue, which is also involved in sterol biosynthesis (*9*). Like Ncpr1p, Erg11p is attached to the membrane by an N-terminal anchor, but unlike Ncpr1p, Erg11p could not be produced as a soluble derivative by expression of an N-terminal truncated mutant. Instead, introduction of a protease recognition site made accessible by flanking diglycines was a route allowing successful generation of soluble enzyme. Similar approaches can be envisaged for other sterol biosynthetic enzymes and may be useful for crystallography studies in the future. Intriguingly, the demonstration of the reconstitution of a complete soluble Erg11p system allows the prospect of attempting cocrystallization of these sterol biosynthetic proteins and may also apply to other parts of the sterol pathway. It is also interesting to consider if the components of sterol biosynthesis evolved from soluble, ancestral enzymes.

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