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Purification and reconstitution of activity of *Saccharomyces cerevisiae* P450 61, a sterol Δ^{22}-desaturase

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Abstract P450 was purified from microsomal fractions of a strain of *Saccharomyces cerevisiae* which contained detectable P450 despite the disruption of *CYP51A1*. The P450 had a molecular mass of 58 kDa, similar to P450 51A1, and in a reconstituted assay with rabbit NADPH-P450 reductase and dilauryl phosphotidylcholine exhibited activity for conversion of ergosta-5,7-dienol into ergosterol. N-Terminal amino acid sequencing of the purified protein corresponded to the translated sequence of P450 61 which was recently identified during sequencing of chromosome XIII. This allowed the function of this family of P450 to be identified as sterol Δ^{22}-desaturation in the pathway of ergosterol biosynthesis.

Key words: Sterol Δ^{22}-desaturase; Purification; P450 61

1. Introduction

The cytochrome P450 superfamily catalyse a wide range of diverse monooxygenase reactions including endogenous and xenobiotic substrates [1]. Eukaryote P450s are primarily located in the endoplasmic reticulum and of the range of P450 activities, one is common to animals, plants and fungi, that is sterol 14α-demethylase [2]. Studies on P450 from *Saccharomyces cerevisiae* identified a single form of P450 following purification from cells grown under semi-anaerobic conditions [3] exhibiting sterol 14α-demethylase activity, but confirmation of the presence of an additional P450 in significant quantities in vegetative yeast arose from studies on strains containing a gene disruption for sterol 14α-demethylase (P450 51A1). Microsomal fractions of this strain were analysed and found to contain P450 [4]. Another P450 gene had been identified in *Saccharomyces cerevisiae* involved in dityrosine formation in sporulating cells [5], but this was sporulation specific and unlikely to be that observed during vegetative growth. Carbon monoxide inhibition studies and the use of the P450 inhibitors metapyrone and SKF525A have demonstrated another step in ergosterol biosynthesis was likely to be undertaken by a P450, one of the final steps in ergosterol biosynthesis involving 22-desaturation [6]. The introduction of the double bond to produce ergosterol, rather than ergosta-5,7-dienol (Fig. 1), appears important for membrane function as indicated by reduced viability of yeast containing such sterol when confronted by osmotic stress.

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In this study we undertook the purification of the residual P450 of vegetative yeast from the strain lacking sterol 14α-demethylase, examined its spectral properties and reconstituted activity. This enabled the identification of its sterol 22-desaturase activity and allowed the function of the recently identified P450 61 family to be established.

2. Materials and methods

2.1. Strains

Saccharomyces cerevisiae strain DK2 was used [4] as the source of microsomal P450. It is a haploid strain containing a gene disrupted sterol 14α-demethylase (*ΔCYP51A1*; *erg11*) and a suppressor mutation in sterol $\Delta^{5,6}$-desaturase (*erg3*) which allows the sterol 14α-demethylase disruptant to grow aerobically by changing the sterol accumulating to 14α-methylfecosterol [4]. Additional auxotrophic markers include *leu 2,3,2–112 trp 1–289 his3-Δ1*.

2.2. Media and cultivation

The strain was grown on complex medium containing 10% (w/v) glucose, 2% (w/v) Difco bacto-peptone and 1% (w/v) Difco yeast extract. Cultures were grown at 30°C and plate cultures contained 2% (w/v) Difco Bacto-agar and only 2% (w/v) glucose. In preparation of biomass for enzyme purification yeast was grown in 2 litre cultures, contained in 3 litre flasks, and shaken at 150 rpm. Cells were inoculated at 10^6 cells/ml and harvested at 5×10^8cells/ml.

2.3. Chemicals

Unless specified all chemicals were obtained from Sigma Chemical Co., Poole, Dorset.

2.4. Cell breakage and protein purification

Cells were harvested at 5×10^8 cells/ml and biomass from a 2 litre culture resuspended in 25 ml of buffer 1 (20% (w/v) glycerol 100 mM potassium phosphate 1 mM EDTA, pH 7.2). 20 g of glass beads were mixed with the cells and they were homogenised using a Braun MSK homogenisor (Braun GmbH) operating at 4000 rpm with 4 × 30 s bursts and liquid carbon dioxide cooling. Cell debris, unbroken cells and glass beads were recovered by centrifugation at 1500 × *g* for 10 min. All steps after breakage were undertaken at 4°C. Mitochondria were removed by centrifugation at 10,000 × *g* for 20 min, followed by a 100,000 × *g* spin for 1 h to produce the microsomal pellet containing P450. The microsomal P450 was resuspended by homogenisation in buffer 1 to a final concentration of protein of approximately 10 mg/ml and microsomal suspensions were stored at −80°C until use. Protein concentration was estimated using a Sigma BCA kit and P450 concentration was determined by reduced carbon monoxide difference spectroscopy according to Omura and Sato [7] using a Philips PU8800 scanning spectrophotometer.

50 nmol of microsomal P450 was solubilised in 25 mM potassium phosphate buffer, pH 7.2, containing 1% (w/v) sodium cholate for 1 h at 4°C. Solubilised P450 was recovered after a 1 h centrifugation at 100,000 × *g* and diluted with a 20% (w/v) glycerol solution to 25 mM potassium phosphate, 0.8% (w/v) sodium cholate.

The supernatant was applied to an amino-octyl agarose column equilibrated with a 10 mM potasssium phosphate, pH 7.2, buffer con-

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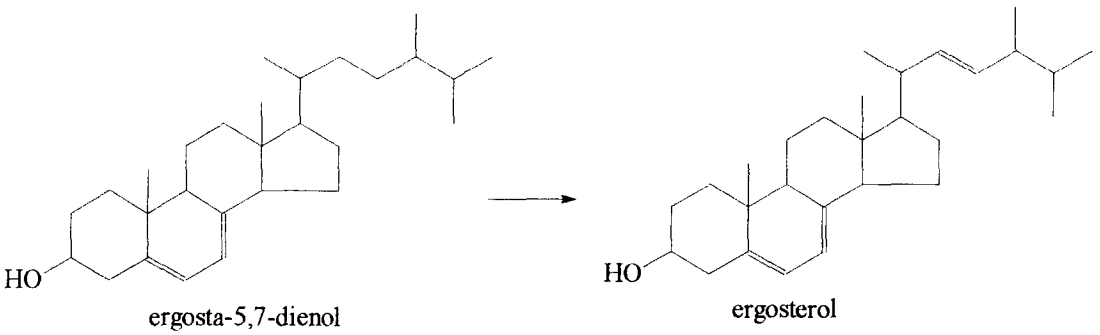


Fig. 1. The conversion of ergosta-5,7-dienol to ergosterol. In the normal pathway 22-desaturation may precede the 25(28)-reduction.

Table 1
Summary of the purification of P450 from microsomes obtained from the *S. cerevisiae* strain DK2

Step	Total content (nmol)	Specific content (nmol/mg)	Yield (%)
Microsomes	50	0.8	100
AO-agarose eluate	28	8.0	56
Bio-gel HT eluate	10	16.5	36

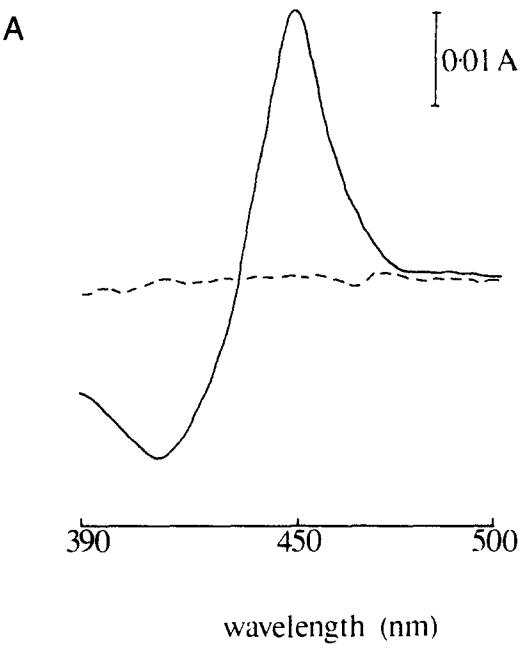
taining 0.8% (w/v) sodium cholate. The column was washed with 100 ml of 10 mM potassium phosphate buffer, pH 7.2, containing 0.8% (w/v) sodium cholate, by a second 200 ml wash with a similar buffer containing 1.2% (w/v) sodium cholate and a third wash with 100 ml of 100 mM potassium phosphate buffer containing 0.5% sodium cholate. P450 was eluted from the column with 100 mM potassium phosphate, pH 7.2, buffer containing 0.5% (w/v) sodium cholate and 0.5% (v/v) Tween 20. P450 containing fractions were pooled and dialysed overnight against 1 litre of 10 mM potassium phosphate buffer, pH 6.8, containing 0.3% (w/v) sodium cholate. The sample was then loaded onto a hydroxylapatite column equilibrated with 10 mM potassium phosphate pH 6.8. The column was washed with 10 mM potassium phosphate, pH 6.8, and eluted using a 10–150 mM phosphate gradient. P450-containing fractions (assessed by reduced carbon monoxide difference spectroscopy) were pooled, concentrated and enzyme purity assessed by SDS-PAGE and specific content. Purified enzyme was stored at −80°C until use.

2.5. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli [8] using a Mini-protean Biorad apparatus and carbonic anhydrase (M_r = 29,000), egg albumin (M_r = 45,000), bovine albumin (M_r = 66,000) and rabbit phosphorylase C (M_r = 97,400) as molecular mass standards.

2.6. Reconstitution of sterol Δ^{22}-desaturase activity

Each reaction mixture contained purified P450 (0.5 nmol) and 1 U of rabbit NADPH-cytochrome P450 reductase (a gift from Prof. M. Akhtar) in a total volume of 50 μl. To this, 50 μg dilauroylphosphatidylcholine (DLPC) was added and the reaction volume adjusted to 950 μl with 100 mM potassium phosphate buffer, pH 7.2. Ergosta-5,7-dienol was added at the appropriate concentration and the mixture



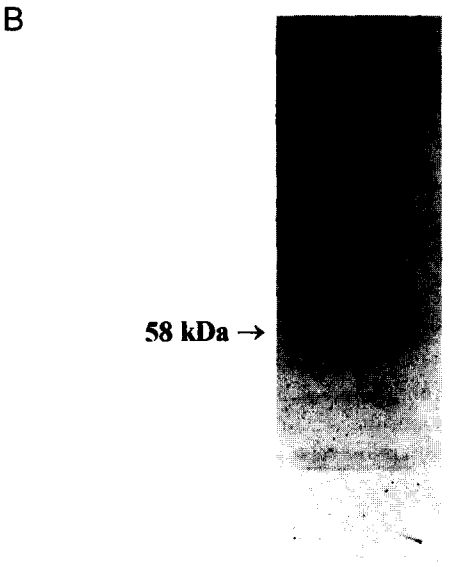


Fig. 2. (a) Reduced carbon monoxide difference spectrum of P450 purified from DK2. (b) SDS-PAGE analysis of purified P450 (2 μg) from *S. cerevisiae* strain DK2 using a 10% polyacrylamide gel and staining the protein with Coomassie blue.

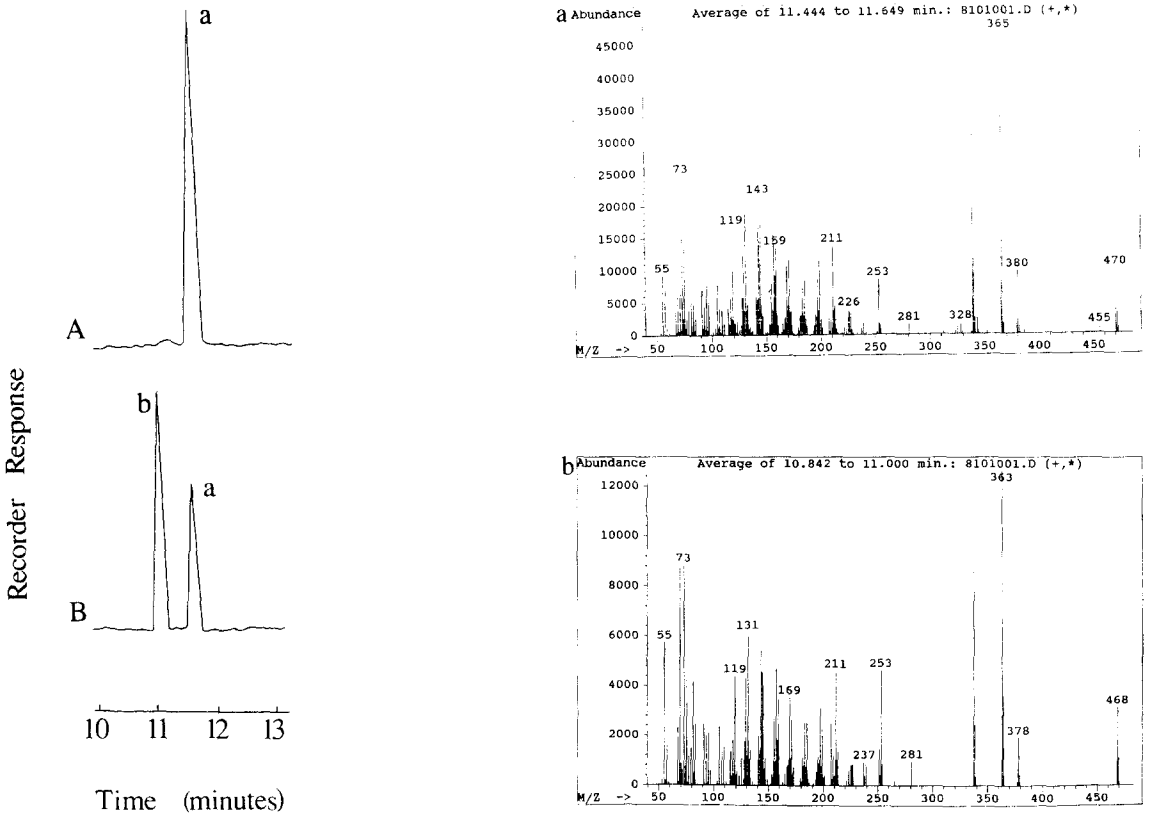


Fig. 3. Gas chromatography of substrate (a) and after reconstitution of P450 activity (b) showing the conversion of ergosta-5,7-dienol to ergosterol, together with characteristic mass spectra.

sonicated until a white suspension had formed. Ergosta-5,7-dienol was purified from a polyene-resistant *erg5* mutant of *S. cerevisiae* [9,10]. NADPH was added at a concentration of 23 mM to the mixture to start the reaction. All reactions were incubated at 37°C for 2 h in a shaking water bath. In control experiments the involvement of P450 was examined by bubbling carbon monoxide through the enzyme preparation prior to substrate addition for 2 min and also by not adding P450 or NADPH to the reconstituted system.

Following the reconstitution of the P450 system as described above sterols were extracted using 3 ml of methanol, 2 ml (0.5% w/v) of pyrogallol in methanol and 2 ml of 60% potassium hydroxide (in water), incubated at 90°C for 2 h in a preheated water bath. After cooling the saponified mixture was extracted with 3 × 5 ml hexane and dried under nitrogen. A Hewlett/Packard GC/MS was used to confirm sterol identities. An Ultra 1 capillary column was used (10m × 0.2 i.d.) on a temperature programme 50°C (1 min) increased by 40°C/min to 290°C with a run time of 17 min. Injection port temperature was 280°C (splitless) and the carrier gas was helium at 40 kPa.

2.7. N-Terminal amino acid sequencing

The amino acid sequence of purified P450 blotted onto PVDF membranes were determined by sequential Edman degredation using an ABI 476A sequencer. Phenylthiohantoin derivatives were analysed with an on-line detector calibrated with 12 pmol of each PTH amino acid.

3. Results

Microsomal P450 from strain DK2 exhibited a reduced carbon monoxide difference spectrum with a peak at 448 nm [4] as is typical of yeast strains with a wild-type *CYP51A1*. When compared to DK1, an isogenic strain from the same tetrad used in the disruption, but lacking the *P450 51A1* disruption, no difference was detected in the specific content of P450. Following growth in 10% (w/v) glucose the content of P450 in DK2 was 82 ± 3 pmol/mg microsomal protein in comparison to 85 ± 6 pmol/mg microsomal protein for the isogenic sister strain retaining P450 51A1 and isolated from the same tetrad dissection.

50 nmol microsomal P450 was accumulated from 20 litre culture and purification undertaken. The results are presented in Table 1 indicating a 25% yield was obtained after both chromatography steps with a specific content of 16.5 nmol/mg protein for the pure protein. The reduced carbon monoxide difference spectrum of the pure protein is shown in Fig. 2a with a maximum apparent at 448 nm. Upon SDS-PAGE, the purified preparation gave a single protein band with an apparent M_r of 58,000 (Fig. 2b).

The reconstituted assay for metabolism of ergosta-5,7-dienol by the purified P450 was analysed by capillary GC analysis of sterol extracts from the reaction (Fig. 3). The identity of the sterol peaks was confirmed by mass spectrometry and showed conversion of the substrate into ergosterol. Control experiments showed complete carbon monoxide inhibition and P450 and NADPH dependancy for the reaction.

Amino acid sequence determination of the N-terminus of the P450 yielded the sequence 5′-TAFSILDXLKS-3′. The order of

MSSVAENIIQHATHNSTLHQLAKDQPSVGVT**TAFSILDTLKS**

Fig. 4. The translation of the DNA sequence assigned to P450 family 61 with the corresponding N-terminal region of the purified protein in bold.

these amino acids was observed for a yeast P450 which has been assigned to the P450 family 61 ([13]; Nelson, personal communication). This was identified during DNA sequencing of chromosome XIII and the amino acid sequence determined here corresponded to residues 32–42 of the open reading frame from the first ATG identified previously (Fig. 4) [13].

4. Discussion

The purification of residual P450 from *S. cerevisiae* has been undertaken and shown to be active in sterol Δ^{22}-desaturation. The characteristics are similar to that reported previously by Yoshida and Aoyama for sterol 14α-demethylase [3] in that firstly they have a reduced carbon monoxide difference Soret peak at 448 nm and secondly the apparent molecular mass appears to be the same, 58,000 on SDS-PAGE.

The P450 purified was active in sterol Δ^{22}-desaturation and this is the endogenous function of the protein. The specific content of P450 in the microsomal fraction of vegetatively growing DK2 was unaltered from that observed without gene disruption of *CYP51A1* suggesting either that the regulation of P450 production was altered or that the sterol 14α-demethylase comprised a minor part of the microsomal P450 when grown under the conditions used. The correlation of the N-terminal sequence of the P450 purified here to a peptide sequence present in the P450 61 sequence deposited recently links the family to an endogenous function. The reason the N-terminus begins with the thirty-second amino acid is unclear. Possible explanations include post-translational modification, although normally only removal of methionine might be anticipated, or degradation of the protein. The extent of the consensus in a non-conserved region of P450s makes us conclude that the purified protein and P450 61 are the same.

Studies are now beginning which will address the importance of sterol Δ^{22}-desaturase for cell survival by gene disruption techniques, the relative proportions and regulation of both sterol biosynthesis P450s under different physiological conditions, as well as structure/function studies. P450 51A1 is an important target for antifungal drugs and agrochemicals [12] and information about P450 61 may allow further such developments. P450 61 may also be involved in determining the overall sensitivity of cells to antifungal azole compounds which inhibit P450 51A1. The P450 61 gene can now be identified as one of last genes of ergosterol biosynthesis to be cloned. Sterol 14α-demethylase is possibly the oldest of the P450s being found in animals, plants, fungi and some bacteria [2]. Study on the evolution of 22-desaturase may also be interesting since this biotransformation is unique to fungal sterols and must have arisen at an early stage of their isolation from other Kingdoms.

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