Sterol 22-desaturase, cytochrome P45061, possesses activity in xenobiotic metabolism

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Abstract CYP61 was revealed in the sequencing of the yeast genome on chromosome XIII and was the last member of the CYP superfamily in yeast to be discovered. We show here that besides the housekeeping role in 22-desaturation during ergosterol biosynthesis the enzyme is also that responsible for benzo(a)pyrene metabolism/promutagen activation by yeast in genotoxicity assays. This enzyme may represent an ancestral activity for the superfamily which allowed xenobiotic metabolism for the first time.

1. Introduction

In eukaryotic organisms lipophilic compounds, such as many natural products and synthetic compounds including drugs, carcinogens and pesticides, cannot be excreted unless they are oxidised to more polar compounds. These oxidation reactions are primarily carried out by the superfamily of cytochrome P450-dependent monooxygenases (for review: [1]). Some cytochrome P450s (CYPs) have evolved to fulfil a role in detoxification, while others are involved in biosynthetic reactions leading to the production of sterols, steroids, terpenes, flavenoids, alkaloids and other products. These latter enzymes are associated with tight substrate specificity unlike many of the detoxification enzymes. From an evolutionary viewpoint the discovery that CYP51, sterol 14α-demethylase, was the first family present in different Kingdoms points to sterol biosynthesis (Fig. 1) as an ancient function for the superfamily [2] and we have been interested in the activity of the second CYP enzyme involved in sterol biosynthesis, CYP61, a 22-desaturase [3].

The inherent ability of *Saccharomyces cerevisiae* to metabolise promutagens/procarcinogens has been extensively studied in genotoxicity tests and includes among other compounds benzo(a)pyrene [4]. For detection of mutagenicity physiological manipulation of the tester strains was undertaken to increase endogenous yeast cytochrome P450. However, controversy over this activity has existed and the role of cytochrome P450 has remained unresolved. Yoshida and Aoyama [5] have not observed benzo(a)pyrene binding or metabolism with purified sterol 14α-demethylase (CYP51) which we have confirmed for the human and plant enzymes (unpublished observation). Recently we have purified and characterised the second cytochrome P450 of vegetative *S. cerevisiae*, CYP61, undertaking sterol Δ^{22}-desaturation [3,6]. This activity is present in organisms from different Kingdoms including protozoa, algae, plants and fungi and confers resistance to osmotic stress on organisms containing 22-desaturated sterol in their membranes [7]. Here we show CYP61 to be the benzo(a)pyrene hydroxylase enzyme from yeast, characterise its enzymatic properties and speculate on the importance of acquiring xenobiotic metabolism activities during evolution of the superfamily.

2. Materials and methods

2.1. Materials

Microsomes were prepared and CYP61 was purified from semi-anaerobically grown cells of the *Saccharomyces cerevisiae cyp51* gene disrupted strain DK2 (*ΔP45051*; *erg11*) [5]. Ergosta-5,7-dienol was purified from a polyene-resistant *erg5* mutant of *S. cerevisiae* (Δ^{22}-desaturase defective) as described previously [6]. Unless specified all chemicals were obtained from Sigma Chemical Co., Poole, Dorset.

2.2. Reconstitution of benzo(a)pyrene metabolism

Benzo(a)pyrene hydroxylase activity was measured using the aryl hydrocarbon hydroxylase assay according to the modified method of King et al. [8,10]. CYP61 (0.5 nmol), 1 U NADPH cytochrome P450 reductase, 23 nM NADPH and varying concentrations of benzo(a)-pyrene, dissolved in dimethylsulphoxide (DMSO) were incubated at 37°C, for 20 min. The reaction was terminated by the addition of ice-cold acetone and precipitated protein removed by centrifugation. Aliquots (0.6 ml) of the supernatant were added to 1.4 ml triethylamine (8.5%) in water and this mixture was centrifuged to remove any residual precipitate. Fluorescence was measured at 467 nm excitation, 525 nm emission. Controls using boiled enzyme and without the addition of NADPH were carried out at each benzo(a)pyrene concentration. Assays were calibrated using a quinine sulphate standard with reference to 3-hydroxybenzo(a)pyrene standards according to Nebert and Gelboin [10]. Activity was measured using a Perkin Elmer 3000 fluorescence spectrophotometer.

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

The standard reaction mixture contained purified P45061 (0.5 nmol), 1 U of rabbit NADPH cytochrome P450 reductase, varying concentrations of ergosta-5,7-dienol dispersed in 80 nmol dilauroylphosphatidylcholine (DLPC) and the reaction volume adjusted to 950 μl with 100 mM potassium phosphate buffer, pH 7.2. NADPH was added at a concentration of 23 mM to the mixture to start the reaction. All reactions were incubated at 37°C for 20 min in a shaking water bath. Reactions were stopped by the addition of 3 ml methanol, and the sterols extracted using 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 (10 m×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. Trimethylatedsilylated derivatives of ergosta-5,7-dienol and the Δ^{22}-desaturated metabolite (ergosterol) were clearly separated as two distinct peaks [6]. The conversion ratio was calculated from the areas of the two peaks and the activity (nmol ergosterol formed/min) was obtained from the amount of ergosta-5,7-dienol added and the conversion ratio. Linear regression was

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used in double reciprocal plots of these and other activity measurements.

2.4. Inhibition of reconstituted sterol Δ^{22}-desaturase activity by benzo(a)pyrene

Ergosta-5,7-dienol at varying concentrations was metabolised aerobically by purified CYP61 in the reconstituted system described above. Benzo(a)pyrene at varying concentrations was added to the reconstituted system in DMSO; control assays received DMSO alone. Ergosta-5,7-dienol and its metabolite, ergosterol, were analysed by GC/MS as described above. Values are the means of triplicate determinations.

3. Results and discussion

CYP61 was capable of benzo(a)pyrene metabolism as measured by the production of 3-hydroxybenzo(a)pyrene (Fig. 2). Fig. 3 shows that the CYP61 reconstituted system has a V_{max} of 1.0 nmol 3-hydroxybenzo(a)pyrene formed h^{-1} per nmol CYP61 and a K_M of 50.1 μM. The V_{max} of 1 nmol h^{-1} per

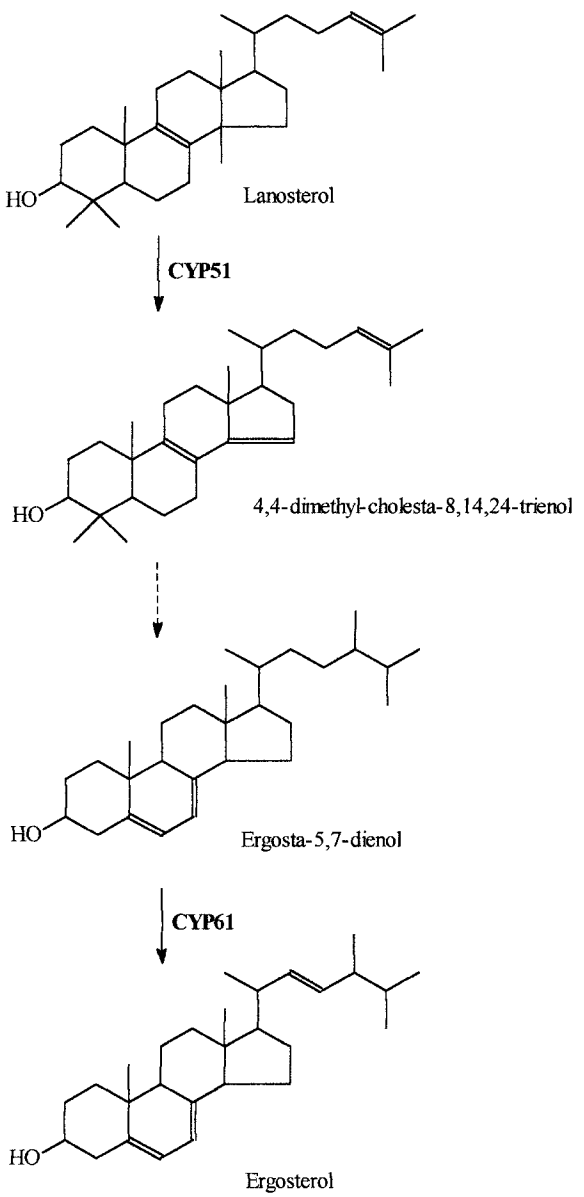

Fig. 1. The biosynthetic steps undertaken by CYP51 and CYP61 during ergosterol biosynthesis.

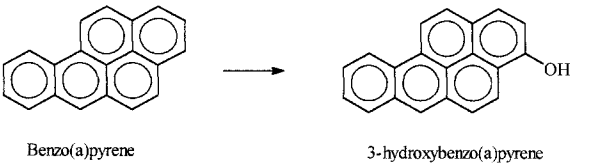

Fig. 2. The cytochrome P450 mediated metabolism of benzo(a)pyrene to 3-hydroxybenzo(a)pyrene.

nmol P45061 was also comparable to the value of 1.1 nmol h^{-1} per nmol P450 obtained by King et al. [8] who purified a benzo(a)pyrene hydroxylase which produced mutagenic metabolites from a brewing strain of yeast, but could not identify an endogenous function for the protein. Benzo(a)pyrene hydroxylation was shown to be dependent on CYP61, NADPH cytochrome P450 reductase and NADPH. Benzo(a)pyrene was also shown to be a competitive inhibitor of Δ^{22}-desaturation (Fig. 4) with the inhibition constant, K_i, determined to be 50.2 μM (IC_{50} = 82 μM).

The successful identification of CYP61 as the benzo(a)pyrene hydroxylase of yeast resolves the question as to the ability of yeast genotoxicity assays to activate procarcinogens. CYP61 is a low activity enzyme operating with a V_{max} about 180-fold lower than for sterol 22-desaturation [3]. The procarcinogens activated include various xenobiotics including aflatoxins, dimethylnitrosamine and various cyclopenta(a) phenanthrenes and the enzyme may be responsible for the rather higher benzo(a)pyrene turnover observed in microsomes of fungi used in bioremediation [9]. Sterol 22-desaturase is the first fungal cytochrome P450 correlated with activity in benzo(a)pyrene metabolism.

Sterol 22-desaturation is found in diverse Kingdoms including algae, protozoa, plants and fungi, but not mammals, whereas CYP51, sterol 14α-demethylase, is present in animals, protists, plants, fungi and probably some bacteria. In considering the evolution of the CYP superfamily, at least in eukaryotes, it appears reasonable to suggest that CYP61 diverged from CYP51 during the first events, conferring osmotic tolerance on primitive unicellular organisms. In acquiring xenobiotic metabolism this may have allowed the initial selection of new enzymes with detoxifying capabilities. Subsequently, the wide array of P450 enzymes seen today may have come about through evolution from CYP61. In animals the need for the endogenous role of the enzyme may have become lost with the emergence of homeostasis or other compensating modifica-

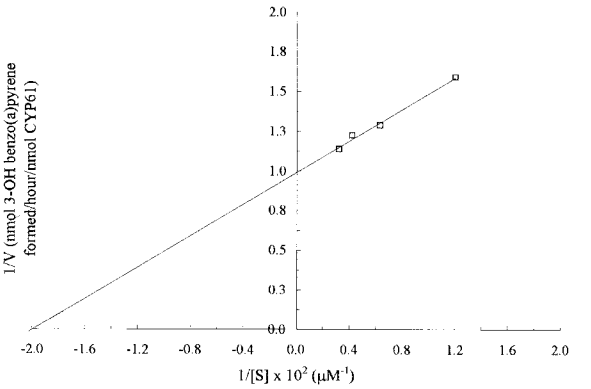

Fig. 3. Double reciprocal plot of benzo(a)pyrene metabolite formation (□) vs. substrate concentration using purified CYP61.

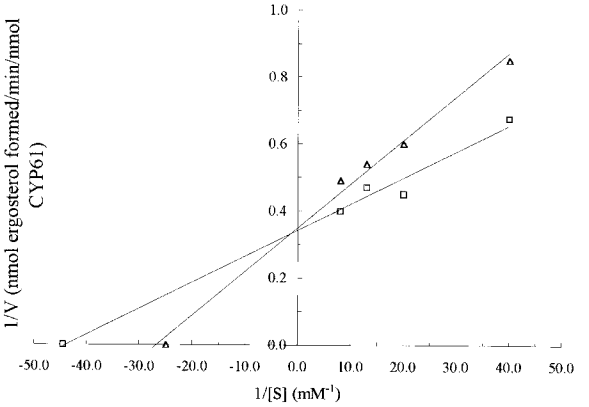

Fig. 4. Effect of benzo(a)pyrene (Δ) on CYP61 mediated ergosta-5,7-dienol Δ^{22}-desaturation. Benzo(a)pyrene was added at half equimolar concentration with respect to CYP61. (□) represents kinetic analysis of endogenous sterol Δ^{22}-desaturation as catalysed by CYP61. Values are the means of triplicate determinations.

tions which alter the requirements for membrane structure/function. Thus we propose that the first step in the production of the xenobiotic metabolising CYP isozymes came about from this CYP51→CYP61 divergent step.

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