

# Molecular diversity of sterol 14 $\alpha$ -demethylase substrates in plants, fungi and humans

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**Abstract** Metabolism of lanosterol (LAN), 24-methylene-24,25-dihydrolanosterol (24-methyleneDHL), dihydrolanosterol (DHL) and obtusifoliosol (OBT) by purified human, plant (*Sorghum bicolor*) and fungal (*Candida albicans*) sterol 14 $\alpha$ -demethylase (CYP51; P450<sub>14DM</sub>) reconstituted with NADPH cytochrome P450 reductases was studied in order to elucidate the substrate specificity and sterol stereo- and regio-structural requirements for optimal CYP51 activity. Both human and *C. albicans* CYP51 could catalyse 14 $\alpha$ -demethylation of each substrate with varying levels of activity, but having slightly higher activity for their respective endogenous substrates in vivo, dihydrolanosterol for human CYP51 ( $V_{\max} = 0.5$  nmol/min/nmol CYP51) and 24-methylene-24,25-dihydrolanosterol for *C. albicans* CYP51 ( $V_{\max} = 0.3$  nmol/min/nmol CYP51). In contrast, *S. bicolor* CYP51 showed strict substrate specificity and selectivity towards its own endogenous substrate, obtusifoliosol ( $V_{\max} = 5.5$  nmol/min/nmol CYP51) and was inactive towards 14 $\alpha$ -demethylation of lanosterol, 24-methylene-24,25-dihydrolanosterol and dihydrolanosterol. These findings confirm that the presence of the 4 $\beta$ -methyl group in the sterol molecule renders the plant CYP51 incapable of 14 $\alpha$ -demethylation thus revealing the strict active site conservation of plant CYP51 during evolution.

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## 1. Introduction

Sterol 14 $\alpha$ -demethylase (CYP51; P450<sub>14DM</sub>) [1,2] is a species of cytochrome P450 which catalyses the oxidative removal of the 14 $\alpha$ -methyl group (C32) of lanosterol and 24-methylene-24,25-dihydrolanosterol in yeasts and fungi, obtusifoliosol in plants and 24,25-dihydrolanosterol in mammals (Fig. 1). This step is considered to be one of the key enzymes of sterol biosynthesis of these organisms and is the target enzyme for the azole antifungals, which are thought to selectively inhibit the yeast and fungal forms over their plant and mammalian counterparts [3].

Previously, the necessary structure of substrate for substrate recognition and catalytic activity of purified *Saccharomyces cerevisiae* CYP51 has been elucidated [4–6]. It was revealed that the sterol ring  $\Delta 8$  double bond and the 3-hydroxyl group were essential for recognition by this CYP51 form. Experiments with partially purified rat CYP51 also showed differences in recognition and affinity for sterol derivatives. Reducing the length of the sterol side chain interfered with substrate binding to rat CYP51, without an effect on  $V_{\max}$ , but the yeast enzyme exhibited reduced catalytic turnover [7]. This suggests fundamental differences in substrate recognition by different CYP51s. Furthermore, studies using corn microsomes have

indicated the C4-demethylated substrate, obtusifoliosol was metabolised, but not C4-demethylated sterols [8]. For plants, obtusifoliosol was thought to be the endogenous substrate in contrast to 24-methylenedihydrolanosterol for fungi and dihydrolanosterol in mammals, but this had not been subject to rigorous analysis using purified enzyme in the case of the plant and fungal (as opposed to yeast) enzyme. In this paper the abilities of the purified CYP51 orthologues from the plant, animal and fungal kingdoms, following heterologous expression, were evaluated for their affinity and C14-demethylation using the three endogenous substrates; obtusifoliosol for *Sorghum bicolor* CYP51, 24-methylene-24,25-dihydrolanosterol for *Candida albicans* CYP51 and dihydrolanosterol for human CYP51 (Fig. 1). Additionally, lanosterol, the substrate for yeast CYP51, was assayed as a potential substrate for all three forms.

## 2. Materials and methods

Human and *C. albicans* CYP51 were expressed in *S. cerevisiae* and purified as previously described [9]. *S. bicolor* CYP51 was expressed in *E. coli* and purified according to the method of Bak et al. [10]. Mammalian, fungal and plant NADPH cytochrome P450 reductases were purified according to the method of Yasuochi and Masters [11]. Lanosterol was purchased from Sigma, Poole, UK. Dihydrolanosterol and 24-methylene-24,25-dihydrolanosterol were gifts from Zeneca Agrochemicals, Berkshire, UK and obtusifoliosol was kindly provided by Rachael Kahn, Plant Biochemistry Laboratory, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. All the sterols used were of the highest purity (not less than 99%) as judged by high performance liquid chromatography (HPLC) and gas chromatography/mass spectroscopy (GC/MS). All other chemicals used were purchased from Sigma, Poole, UK. The 14 $\alpha$ -demethylase activity of the plant, *C. albicans* and human enzymes consisting of CYP51 and its respective reductase was assayed according to previous methods [12,13]. Each sterol was dispersed in dilaurylphosphatidylcholine (DLPC) and added to the reaction mixture at varying concentrations. The reconstituted system contained 0.1 nmol of each purified CYP51 and 1.0 unit of its respective NADPH cytochrome P450 reductase. The activity was assayed by GC/MS determination of the demethylated products. Briefly, the sterols were extracted into heptane, dried down and trimethylsilylated for 1 h at 60°C with BSTFA (50  $\mu$ l) in 50  $\mu$ l toluene, sterol substrates and metabolites were clearly separated and identified by GC/MS (VG 12-250 (VG BIOTECH)). The conversion ratio was calculated from the areas of the two peaks and the activity (nmol demethylated product formed/min/nmol CYP51) was calculated from the amount of substrate added with cholesterol added as an internal standard.

## 3. Results and discussion

### 3.1. Metabolism of lanosterol

Purified human and *C. albicans* CYP51 catalysed the oxidative removal of the C14 methyl group from lanosterol to the product, 4,4-dimethylcholesta-8,14,24-trien-3 $\beta$ -ol. Rate of lanosterol 14 $\alpha$ -demethylation observed under the standard as-

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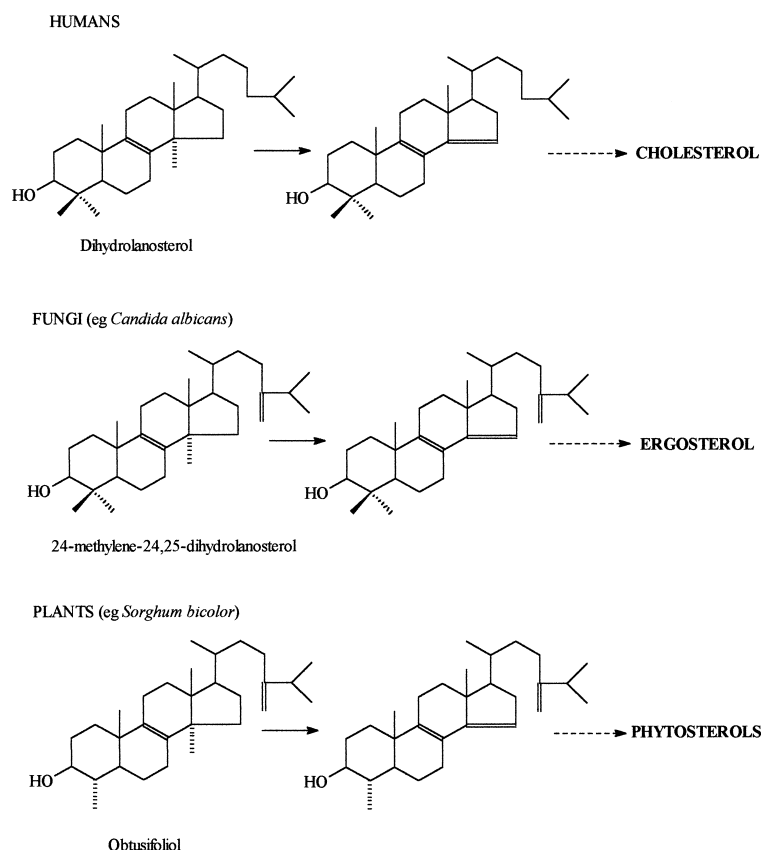


Fig. 1. Eukaryotic CYP51-mediated sterol 14 $\alpha$ -demethylation. In humans, 14 $\alpha$ -demethylation occurs on dihydrolanosterol during cholesterol biosynthesis; in fungi, this reaction occurs on either lanosterol or 24-methylene-24,25-dihydrolanosterol during ergosterol biosynthesis and in plants obtusifolol acts as the CYP51 substrate during phytosterol production.

say conditions as described were 0.25 nmol/min/nmol CYP51 for *C. albicans* and 0.18 nmol/min/nmol CYP51 for humans. This activity was lower than those previously reported for purified *S. cerevisiae* CYP51 (7.7 nmol/min/nmol CYP51 [12]), reflecting the inherent higher activity of the yeast enzyme. Purified plant CYP51 showed no ability to demethylate lanosterol. Additionally, when obtusifolol was added in conjunction to lanosterol to the reconstituted system for the plant enzyme, the presence of the product formed from the demethylation of obtusifolol, 4 $\alpha$ -methylergosta-8,14,24(28)-trien-3 $\beta$ -ol, was seen. These observations indicate that lanosterol is an incompatible substrate for the plant enzyme whereby the structure of this sterol molecule prevents binding to the active site of this cytochrome. Both human and *C. albicans* CYP51 showed similar activity towards lanosterol, which is expected due to the closeness of this sterol to their respective endogenous substrates (Table 1).

### 3.2. Metabolism of 24-methylene-24,25-dihydrolanosterol and dihydrolanosterol

24-methyleneDHL and DHL were also metabolised by the reconstituted lanosterol demethylases of humans and *C. albicans* to their 14 $\alpha$ -demethylated products. 24-methyleneDHL was shown to be a slightly better substrate for purified *C. albicans* CYP51 (0.3 nmol/min/nmol) compared with other sterol substrates used in this study which is consistent with it being the endogenous substrate in *C. albicans*. Conversely, purified human CYP51 had lower activity in its ability to

demethylate 24-methyleneDHL with an observed rate equivalent to 0.22 nmol/min/nmol CYP51. Furthermore, human CYP51 was shown to be most active in its ability to demethylate DHL in these studies (observed activity 0.5 nmol/min/nmol CYP51) whereas *C. albicans* CYP51 showed lower activity in DHL demethylation. Purified plant CYP51 had no activity in 24-methyleneDHL and DHL demethylation in reconstituted systems. Again, when obtusifolol is added to these reconstitutions, 4 $\alpha$ -methylergosta-8,14,24(28)-trien-3 $\beta$ -ol was seen, indicating an inherent inability of plant CYP51 to bind and metabolise other CYP51 substrates from different kingdoms.

Table 1

Kinetic parameters for the sterol 14 $\alpha$ -demethylation of lanosterol, 24-methylene-24,25-dihydrolanosterol, dihydrolanosterol and obtusifolol as catalysed in reconstituted systems containing purified human, *C. albicans* and *S. bicolor* CYP51 and their analogous NADPH cytochrome P450 reductases

Substrate	CYP51 orthologue					
	Human		<i>C. albicans</i>		<i>S. bicolor</i>	
	$K_M^a$	$V_{max}^b$	$K_M^a$	$V_{max}^b$	$K_M^a$	$V_{max}^b$
Lanosterol	29	0.18	15.0	0.25	—	—
24-methyleneDHL	32	0.22	11.0	0.3	—	—
Dihydrolanosterol	27	0.5	25.0	0.12	—	—
Obtusifolol	32	0.44	28.5	0.26	18.0	5.5

<sup>a</sup> $\mu$ M.

<sup>b</sup>Activity is expressed as nmol/min/nmol CYP51.

### 3.3. Metabolism of obtusifoliosol

As described above, purified plant CYP51 converted obtusifoliosol to the 14 $\alpha$ -demethylated product, 4 $\alpha$ -methylergosta-8,14,24(28)-trien-3 $\beta$ -ol, as detected by GC/MS. The rate of obtusifoliosol 14 $\alpha$ -demethylation was high (5.5 nmol/min/nmol CYP51) with the activity unaffected by the addition of lanosterol, 24-methyleneDHL and DHL. Purified human and *C. albicans* CYP51 metabolised obtusifoliosol to its 14 $\alpha$ -demethylated product. However, this activity was lower than those seen for lanosterol, 24-methyleneDHL and DHL. These results indicate that *S. bicolor* CYP51 is highly specific for one type of CYP51 substrate that is found in nature, obtusifoliosol, whereas human and *C. albicans* CYP51 have a wide spectrum of activity towards different CYP51 substrates.

The inability of purified *S. bicolor* CYP51 to metabolise 14 $\alpha$ -demethylate lanosterol, 24-methylene-24,25-dihydrolanosterol and dihydrolanosterol indicates that the presence of a structural feature common to all three sterol molecules, the 4 $\beta$ -methyl group (Fig. 1), prevents binding of these substrates to the plant enzyme, as previously shown with corn microsomes [8]. Additionally, metabolism studies on obtusifoliosol and each other substrate present in the reconstituted system containing purified *S. bicolor* CYP51 presented here reveal only obtusifoliosol to be metabolised, indicating both access of obtusifoliosol to the active site of the plant CYP51 and conversely, the inability of the other substrates to gain access. Such unique substrate specificity of the plant enzyme is unusual when data for the human and fungal enzymes are compared. Both these enzymes could metabolise all four substrates in the present study, with confirmation for the first time that their speculated endogenous substrates are indeed favoured.

CYP51 is the only P450 form found in plants, animals and fungi indicating both the essential role this enzyme plays in eukaryotic cell integrity and also that CYP51s of different organisms originated in a common ancestor [14]. It has been suggested that the earliest role of P450 was as a defence mechanism in order to detoxify oxygen and its reactive intermedi-

ates, which formed during the simple processes of cellular metabolism and photosynthesis in the primitive cell [15], but other more primitive CYP forms may have been involved in this. The reason for the substrate selectivity difference observed for the plant as opposed to human/fungal CYP51s presumably reflects the early divergence of the sterol biosynthetic pathway in the plant kingdom.

### References

- [1] Yoshida, Y. and Aoyama, Y. (1984) *J. Biol. Chem.* 259, 1655–1660.
- [2] Trzaskos, J., Kawata, S. and Gaylor, J.L. (1986) *J. Biol. Chem.* 261, 14651–14657.
- [3] Vanden Bossche, H., Marichal, P., Gorrens, J., Bellens, D., Verhoeven, H.D., Coene, M.C., Lauwers, W.F. and Janssen, P.A.J. (1987) *Pest. Sci.* 21, 289–306.
- [4] Aoyama, Y., Yoshida, Y., Sonoda, Y. and Sato, Y. (1989) *Biochim. Biophys. Acta* 1006, 209–213.
- [5] Aoyama, Y., Yoshida, Y., Sonoda, Y. and Sato, Y. (1989) *Biochim. Biophys. Acta* 1001, 196–200.
- [6] Aoyama, Y., Yoshida, Y., Sonoda, Y. and Sato, Y. (1989) *Biochim. Biophys. Acta* 1081, 262–266.
- [7] Aoyama, Y. and Yoshida, Y. (1991) *Biochem. Biophys. Res. Commun.* 178, 1064–1071.
- [8] Taton, M. and Rahier, A. (1991) *Biochem. J.* 277, 483–492.
- [9] Lamb, D.C., Kelly, D.E., Schunck, W.-H., Shyadehi, A.Z., Akhtar, M., Lowe, D.J., Baldwin, B.C. and Kelly, S.L. (1997) *J. Biol. Chem.* 272, 5682–5688.
- [10] Bak, S., Kahn, R.H., Olsen, C.-E. and Halkier, B.A. (1997) *Plant J.* 11, 191–201.
- [11] Yasuochi, Y. and Masters, B.S.S. (1976) *J. Biol. Chem.* 51, 5337–5344.
- [12] Aoyama, Y., Yoshida, Y. and Sato, R. (1984) *J. Biol. Chem.* 259, 1661–1666.
- [13] Hitchcock, C.A., Dickinson, K., Brown, S.B., Evans, G.V. and Adams, D.J. (1990) *Biochem. J.* 266, 475–480.
- [14] Aoyama, Y., Noshiro, M., Gotoh, O., Imaoka, S., Funae, Y., Kurosawa, N., Horiuchi, T. and Yoshida, Y. (1996) *J. Biochem.* 119, 926–933.
- [15] Wickramasinghe, R.H. and Vilee, C.A. (1975) *Nature* 256, 509–511.