The Mechanism of the Acyl-Carbon Bond Cleavage Reaction Catalyzed by Recombinant Sterol 14α-Demethylase of Candida albicans (Other Names Are: Lanosterol 14α-Demethylase, P-450<sub>14DM</sub>, and CYP51)*

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The Candida albicans sterol 14α-demethylase gene (P-450<sub>14DM</sub>, CYP51) was transferred to the yeast plasmid YEp51 placing it under the control of the GAL10 promoter. The resulting construct (YEpsCYP51) when transformed into the yeast strain GRF18 gave a clone producing 1.5 μmol of P-450/liter of culture, the microsomal fraction of which contained up to 2.5 nmol of P-450/mg of protein. Two oxygenated precursors for the 14α-demethylase, 3β-hydroxylanost-7-en-32-al and 3β-hydroxylanost-7-en-32-ol, variously labeled with 3H and 18O at C-32 were synthesized. In this study the conversion of [32-2H<sub>2</sub>,32-18O] and [32-2H,32-18O]3β-hydroxylanost-7-en-32-al with the recombinant 14α-demethylase was performed under 16O<sub>2</sub> or 18O<sub>2</sub> and the released formic acid analyzed by mass spectrometry. The results showed that in the acyl-carbon bond cleavage step (i.e., the deformylation process) the original carbonyl oxygen at C-32 of the precursor is retained in formic acid and the second oxygen of formate is derived from molecular oxygen; precisely the same scenario that has previously been observed for the acyl-carbon cleavage steps catalyzed by aromatase (P-450<sub>aro</sub>) and 17α-hydroxylase-17,20-lyase (P-450<sub>17α</sub>, CYP17). In the light of these results the mechanism of the acyl-carbon bond cleavage step catalyzed by the 14α-demethylase is considered.

Our earlier studies on the removal of C-19 of androgens in the formation of estrogens (1–3) and of the 14α-methyl group of lanosterol during sterol biosynthesis (Scheme I, Conversion 1→4) (4) raised the possibility that these seemingly unrelated conversions may occur by closely related mechanisms involving three steps as shown in Reaction 1.

These studies also indicated that in each case the same catalyst was responsible for all three reactions, and this feature was firmly established through genetic studies and purification to homogeneity of the two enzymes, aromatase (P-450<sub>aro</sub>) (5, 6) and lanosterol 14α-demethylase (P-450<sub>14DM</sub>) (7, 8). The third step in estrogen biosynthesis has aroused much interest (9, 10) and the current view of the mechanism is influenced by our 18O labeling experiments (2, 3), which highlighted the novel nature of the process, leading to the proposal that the reaction involves an acyl-carbon cleavage represented by Reaction 2 (9).

Although all the experimental findings available to date on the C–C bond cleavage step in 14α-demethylation, for example the requirement for NADPH plus O<sub>2</sub> for the reaction and release of the C<sub>1</sub> unit as formate, could be explained (3, 9) by the reaction of Reaction 2, the direct scrutiny of the hypothesis has not been possible hitherto due to the unavailability both of appropriately labeled 18O substrates and an enzyme preparation that produced sufficient formic acid for accurate 18O isotope analysis.

The present paper describes a satisfactory resolution of these difficulties and reports on the status of oxygen during the C–C bond cleavage step catalyzed by lanosterol 14α-demethylase (3→4).

**EXPERIMENTAL PROCEDURES**

Materials—Isotopically enriched 18O<sub>2</sub> (97%) admixed with 2 volumes of argon was obtained from Isogas Limited, Croydon, Surrey and H<sub>2</sub>18O was from MSD Isotopes, Montreal, Canada. Dry redistilled solvents were used, and the petroleum ether used was that with a boiling range of 60–80 °C. Diazotoluene was prepared from N-benzyl-N-nitrosotoluene-4-sulfonamide (11). The phrase "in the usual manner" indicates that the reaction mixture was poured into water, the product extracted with ethyl acetate, the combined organic extracts washed with water, dried over anhydrous sodium sulfate, and the solvent removed under reduced pressure. All the intermediates used for the synthesis of 3β-acetoxylanost-7-en-32-nitrile (5) gave expected melting points, R<sub>f</sub> values, IR spectra, as well as mass spectrometric data.

Gas Chromatography-Mass Spectrometry—Isotopic distributions in the labeled substrates were determined by direct introduction probe mass spectrometric analyses of either the underivatized materials or their trimethylsilyl derivatives and are corrected for 13C natural abundance. All the mass spectra were recorded in the electron impact positive ion mode.

The analysis of benzyl formate, prepared from the enzymatically produced formic acid, was performed by gas chromatography-mass spectrometry using a Hewlett-Packard 5890/ VG TS-250 and a 30 m × 0.32 mm inner diameter column of DB17 with splitless injection (12). The experimentally determined value for 13C natural abundance (12.4%) for benzyl formate was used to correct all the peaks between m/z 137–141, due to other isotopomers. The distribution of the isoto- pomers in benzaldehyde was measured by comparison of the normal- ized ion signal area determined by selected ion recording and corrob- orated by recording the full spectrum.

3β-Hydroxylanost-7-en-32-al (6a)—A commercially available mixture of lanost-8-en-3β-ol and lanost-8,24-dien-3β-ol (approx. 1:1) was...
The trimethylsilyl derivative of the latter gave 30. The trimethylsilyl derivative of the latter gave m/z 444. [32-H]-3-hydroxylanost-7-en-3-ol (7a)—To 3-hydroxylanost-7-en-3-ol (6a), 84 mg, 0.019 mmol dissolved in methanol (6 ml) and tetrahydrufuran (5 ml; 30 cm; petroleum ether) was added sodium borohydride (25 mg, 0.62 mmol), and the solution was allowed to stand at room temperature for 9.5 h. The product was isolated in the usual manner and chromatographed on a silica gel column (2 x 38 cm) with petroleum ether containing increasing amounts of ethyl acetate (up to 20%) to give after crystallization from diethyl ether-petroleum ether 3-hydroxylanost-7-en-3-ol (70 mg, 0.16 mmol, m.p. 200–203°C, Rf 0.62 (ethyl acetate-petroleum ether, 1:1) and its mass spectrum gave a molecular ion at m/z 444. [32-H]-3-hydroxylanost-7-en-3-ol (7b)—This compound was prepared from [32-H]-3-hydroxylanost-7-en-3-ol (6b) as for the unlabeled compound (7a), but utilizing sodium borodeuteride. The recrystallized product had m/z 444 (indicated by italics) followed by “composition; percent distribution” (indicated inside the parentheses) 446 (D2; 78%), 445 (D1; 13%), and 444 (D1; 12%). [32-H]-3-hydroxylanost-7-en-3-ol (7c)—A solution of 3-hydroxylanost-7-en-3-ol (6a), 90 mg) in methanol (6 ml) and tetrahydrufuran (3 ml) was first treated with sodium borotritide (2 mg; 3–5 mCi) for 0.5 h and then unlabeled sodium borohydride (25 mg) for another 0.5 h. The reaction mixture was worked up, in the usual manner, to give 7d (14.6 μCi/μmol).

Preparation of Microsomes from Pig and Rat Liver—Pig or rat livers were cut into small pieces and suspended in approximately 2.5 times their volume of 100 mM potassium phosphate (also containing 2 mM glutathione, 1 mM EDTA, 4 mM magnesium chloride, 0.25 M sucrose, 0.25 mM phenylmethylsulfonyl fluoride, pH 7.4) and homogenized. The homogenate was centrifuged at 10,000 x g for 30 min and the supernatant was subsequently spun at 10,500 x g for 1.5 h twice. The resulting microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (1 mM glutathione, 1 mM EDTA, pH 7.4) to give a final concentration of 40–60 mg ml⁻¹ protein.

Recombinant DNA Manipulations—Our previous studies have employed a yeast expression system to express the Candida albicans CYP51 using the Saccharomyces cerevisiae phosphoglycerate kinase promoter in vector pW91P (15). Higher level expression was achieved by recombinant PCR to allow transfer of CYP51 to YEp51 on a S. cerevisiae host. Inside primers used in the PCR mutagenesis were: 1, 3'-AAAGAAATTAAATCTAGAA-3' and 2, 5'-CGAGATGAATGCGC-3'. The primer sequences were cloned directly into S. cerevisiae vector pW91P on the NsiI dIII fragment containing the endogenous NsiI site present in the gene. Recombinant PCR was used to replace the triple 263 (CTG) with one encoding serine (TCT) in the S. cerevisiae host. Inside primers used in the PCR mutagenesis were: 1, 5'-TAAGAAGATATATCTGAA-3' and 2, 5'-ACGTTCCTCCATTAGT-3' annealing to the 3' end at position 1098–1125 of the CYP51 and containing the endogenous NsiI site present in the gene. Recombinant PCR was used to replace the triple 263 (CTG) with one encoding serine (TCT) in the S. cerevisiae host. Inside primers used in the PCR mutagenesis were: 1, 5'-AAAGAAATTAAATCTAGAA-3' and 2, 5'-ACGTTCCTCCATTAGT-3'.

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of the 32-oxo (6) and 32-hydroxy (7) substrates. The main methodological improvement made in the synthetic protocol (Scheme II) was the use of diisobutylaluminum hydride, instead of LiAlH₄ in the original work for the conversion of the nitrite (5) into aldehyde (6), which decreased the reaction time from 72 to less than 0.5 h.

The two tritiated substrates (6e) and (7d) were used for the assay of the 14α-demethylase activity by monitoring the release, in the medium, of HCOOH from the aldehyde (6e) or HCOOH plus H₂O from the hydroxy compound (7d). In the metabolism of the hydroxy compound (7d), tritium is released in water during the oxidation of the hydroxy into the aldehyde group and in formic acid during the subsequent C–C bond cleavage step converting the 32-oxo derivative (6) to the 7,14-diene (see Scheme III, structure of the type 11). An oxidative activity in most preparations of 14α-demethylase converts the initially produced formate into CO₂ and H₂O. Our projected mechanistic experiments required an improved enzyme activity, free from the above oxidation reaction, in order to provide at least 4 μmol of formic acid for MS analysis.

Heterologous Expression of Sterol 14α-Demethylase of C. albicans—The requirement for an improved source of enzyme for the projected study and the importance of sterol 14α-demethylase as a target for the development of antifungal agents prompted experiments on the expression of the enzyme. In our previous studies the vector pW91P containing phosphoglycerate kinase promoter was used for the expression of C. albicans CYP51 gene in S. cerevisiae, and about 100 pmol of enzymatically active microsomal protein were obtained (18). Further improvement has now been achieved using GAL10 promoter (19) of the vector YEp51 in conjunction with the yeast strain GRF18 (20). Under the conditions of growth used in the present study, GRF18 harboring the expression vector without the insert gave undetectable levels of P-450; however, the cells still synthesized ergosterol, indicating a low level of endogenous expression. Although the phosphoglycerate kinase expression system had indicated that functional C. albicans CYP51 is produced from the native gene (18), we rectified the mutation that will occur on expression in S. cerevisiae due to the deviation in the genetic code discovered in C. albicans (21). In the latter organism, CTG, the triplet for Leu, is used for the incorporation of Ser. The alteration of the CTG triplet at position 263 to TCT by recombinant PCR was undertaken to allow a Ser to be inserted in this position, as occurs in C. albicans, when the protein is expressed in S. cerevisiae instead of Leu. The cloning strategy is illustrated in Fig. 1 and other details are described under “Experimental Procedures.”

Transformation of the yeast strain GRF18 with YEp51: CYP51 produced 1.5 μmol of the demethylase/liter of culture, while the derived microsomal fraction was found to contain up to 2.5 nmol of P-450/mg of protein. The level of expression is
higher than has been reported for other P-450 in yeast or E. coli, suggesting that the availability of heme is not limiting. This productivity was not dependent on the CTG to TCT mutation undertaken. Molecular modelling studies predict that the residue at position 263 is on the surface of the protein, thus explaining the absence of effect on the activity of the enzyme when the unmodified gene was expressed previously.2

Table I shows that the specific activity of the enzyme in microsomes from recombinant vector, based on release of formic acid from the 3H-labeled 32-oxo derivative (6e), was 0.1-0.25 nmol/nmol of P-450/min, and, as expected, no activity was detectable in the host strain harboring the parent vector. The specific activity of the cloned enzyme remained unchanged when it was purified to homogeneity and reconstituted with NADPH-cytochrome P-450 reductase from pig liver. The activity is similar to that found for homogeneous CYP51 obtained from a wild type strain of C. albicans (22), but lower than those reported from other sources (8, 23–25). The reason for the low specific activity of C. albicans CYP51 is not known but the possibility has been considered that the physiological substrate for this enzyme may be 24-methylenedihydrolanosterol rather than the lanosterol derivatives used in in vitro assays by us and others (22).

Mass Spectrometric Analysis of Formate Released from the 32-Oxo Derivatives (6) Variously Labeled at C-32 with 2H and 18O—Before dealing with the results of the enzymic incubations, attention is drawn to the fact that a quantitative determination of the 18O content of formate produced biosynthetically is fraught with a number of problems. The one that should

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14α-Demethylase activities of various microsomal fractions

Assays were performed as described under "Experimental Procedures" by monitoring the release of water-soluble 3H from either [32-3H]3β-hydroxylanost-7-en-32-ol (aldehyde, 6e) or [32-3H]3β-hydroxylanost-7-en-32-ol (hydroxy, 7d).

| Source of microsomal fraction | Substrate       | pmol of substrate converted/mg of protein/min |
|-------------------------------|-----------------|---------------------------------------------|
| Rat liver                     | Aldehyde (6e)   | 52                                          |
| Pig liver                     | Aldehyde (6e)   | 20                                          |
| Baker’s yeast                 | Aldehyde (6e)   | 28                                          |
| GRF18 containing YEp51:CYP51  | Hydroxy (7d)    | 82a                                         |
| GRF18 containing YEp51:CYP51  | Aldehyde (6e)   | Undetectable                                |

*The activity expressed as nmol of substrate converted/min of P-450/min in different preparations was 0.1–0.25.

Isotopic analysis of benzyl formate derived from variously labelled isotopomers of 3β-hydroxylanost-7-en-32-ol incubated under 16O2 or 18O2 Incubations were performed and processed as described under "Experimental Procedures." Only the deuterium and 18O containing isotopomers of benzyl formate are included in the calculation of the data. The unlabeled benzyl formate originating from formic acid ubiquitously present in reagents and enzyme preparation has the molecular ion peak at m/z 136 the intensity of which was used to correct for 13C natural abundance from all the peaks between m/z 137–141. D = 2H; O = 18O and U denotes the unlabeled species.

| substrate and isotopic composition | gas phase | relative intensities of peaks due to the benzyl ester of |
|-----------------------------------|-----------|--------------------------------------------------------|
| D1(80%);U(20%)                    | 16O2      | DCOOH (m/z 137) HCOOH (m/z 138) HCOOH (m/z 139) DCOOH (m/z 140) |
| 1. (6b)                           |           | 100 0 0 0 0                                              |
| D1(80%);U(20%)                    | 18O2      |                                                         |
| 2. (6b)                           |           | 18 21 50 11 0                                           |
| D1(80%);U(20%)                    | 16O2      |                                                         |
| 3. (6c)                           |           | 12 0 0 0 0                                              |
| D1,18O(70%);18O(6%);D1(7%);U(17%) | 18O2      |                                                         |
| 4. (6c)                           |           | 28 6 22 3 41                                            |
| D1,18O(70%);18O(6%);D1(7%);U(17%) |           |                                                         |
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DISCUSSION AND CONCLUSIONS

The notion that the C–C bond cleavage reaction in the multistep process catalyzed by 14α-demethylase occurs by the same generic reaction that has previously been found to operate for aromatase (CYP19) (2, 3) and 17α-hydroxylase-17,20-lyase (CYP17) (12) is supported by the present study. The fission process corresponding to an acyl-carbon cleavage is reducible to the stoichiometry of Reaction 2. The two main predictions of Reaction 2, that in the overall process the carbonyl oxygen atom of the substrate, together with an atom of oxygen from O₂, are incorporated in the expelled formate, have been validated experimentally. We have advocated that the cleavage process may be rationalized by assuming that in the catalytic cycle of P-450s, the FeIII-OOH species, which is normally directed to produce an iron-monoxygen species involved in the hydroxylation reaction, may be trapped to give an adduct when the substrate skeleton contains a properly juxtapositioned electrophilic center (26, 27).

The intermediacy of a peroxide adduct in acyl-carbon bond cleavages (Reaction 2) has been inferred from a range of observations, either described or reviewed in previous publications (10, 27–30). Several mechanistic alternatives are possible through which the products of the reaction of Reaction 2 may be formed from the peroxide-adduct of the type B, Scheme III. For example, in the case of CYP17 evidence has been presented to show that certain acyl-carbon bond cleavage reactions catalyzed by the enzyme occur by a homolytic fission route producing a carbon radical that either undergoes a disproportionation process producing an olefin or an oxygen rebound reaction forming a hydroxy compound (29). A similar scenario may be envisaged for the related acyl-carbon bond cleavage reaction, promoted by 14α-demethylase, as shown in Scheme III. When the intermediacy of a peroxide-adduct was originally proposed, the possibility was considered that it may rearrange by a Baeyer-Villiger process to produce a formate ester which then, through an elimination reaction, creates the double bond in the final product (2). Such a possibility was, however, excluded for aromatase by showing that 10β-hydroxyestr-4-ene-3,17-dione formate was not aromatized by the enzyme (2, 9). O-Acyl derivatives have been isolated during the reactions catalyzed by 14α-demethylase (31) and CYP17 (32), but further work is required to establish whether these are bona fide intermediates in the acyl-carbon cleavage reaction or are formed merely as side products. The problem posed by a mechanism for the reaction of Reaction 2, which operates through the intermediacy of an O-acyl derivative is that it requires a single enzyme, not only to possess the activity for three different oxidative reactions, but also a fourth activity to promote the difficult removal of the elements of formic acid.

In the light of these considerations, we favor the mechanism shown in Scheme III for the conversion of the 32-oxo derivative (6) into the 7,14-diene (11). In principle the initially formed peroxo adduct (8) may cleave by an ionic or a radical process. The latter cleavage mode, however, has the advantage that it gives an intermediate alkyo radical (9), which is ideally suited to undergo fragmentation producing formate, and the substrate radical (10), which can be conveniently converted into the product (11). Furthermore, the mechanism is based on a precedent from an equivalent acyl-carbon cleavage reaction catalyzed by CYP17 for which evidence for a radical process has been obtained (29).

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