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PURIFICATION OF A TERMINAL OXYGENASE IN DEMETHYLATION OF C-30 OF LANOSTEROL

U. S. Maitra, V. P. Mohan, H. Kochi[1], V. Shankar[2]
M. Adlersberg, K.-P. Liu[3], L. Ponticorvo, and D. B. Sprinson[4]

Department of Biochemistry, College of Physicians and Surgeons, Columbia University; and Department of Medicine, St. Luke's-Roosevelt Hospital Center, 428 West 59th Street, New York, N. Y. 10019

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SUMMARY: Oxygenation of the α-methyl group at C-4 (C-30) of lanosterol in solubilized rat liver microsomes was studied by ^3H release from [C-30-^{3}H]-4, 4'-dimethylzymosterol. This activity required oxygen, a cyanide sensitive non-heme iron protein which was purified to homogeniety, either NADH or NADPH, and a labile fraction which has resisted resolution or purification. Attempts to implicate cytochrome P-450 reductase, cytochrome b_5 reductase, or cytochrome b_5 as electron transfer proteins in this system were not successful, suggesting that a new redox protein may be involved in oxygenation of C-30 of lanosterol.

In the biosynthesis of cholesterol and ergosterol from lanosterol (Fig. 1, I) the α-methyl group at C-14 (C-32) is removed first as formate. The methyl groups at C-4 are removed as CO_2: first the α-equatorial substituent (C-30), and then β-axial (C-31) (1-3). Oxygenation and removal of C-32 in microsomes from semi-anaerobically grown yeast was effected by cytochrome P-450 which was purified to homogeneity and required for activity NADPH as a source of electrons and cytochrome P-450 reductase

1. Career Investigator Visiting Scientist of the American Heart Association, 1975-1977. Present address: Department of Biochemistry, Fukushima Medical College, Fukushima, Japan.
2. Present address: Department of Biochemistry, National Chemical Laboratory, Poona India.
3. Present address: Division of Neuroscience, New York State Psychiatric Institute, New York, N. Y. 10032
4. Career Investigator of the American Heart Association during part of this work.

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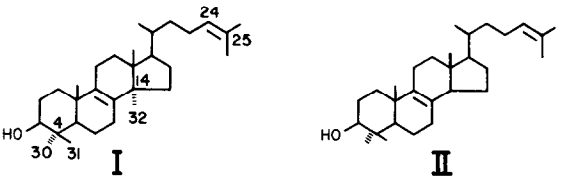

Fig. 1. Lanosterol (I) and 4, 4'-dimethylzymosterol (II). When prepared from [2-^{3}H]RS-mevalonolactone (manuscript in preparation) II is labeled at C-1, 7, 15, 22, 26, and 30. In the conversion of II to cholesterol, oxygen-dependent release of ^{3}H occurs only from C-30 (1). Hence labeled II is prefixed for convenience with " C-30-^{3}H " in the text.

(4). Removal of C-30 of lanosterol in yeast microsomes was inhibited by cyanide (5, 6), and required cytochrome b_5 (6). A requirement for cytochrome b_5 in C-30 removal was also reported in liver microsomes and microsomal extracts (7).

Our interest in lanosterol demethylation was stimulated by the discovery of prototrophic yeast mutants defective in removal of C-32 of lanosterol, but capable of removing C-30 and C-31 (8, 9). In the present study oxygenation of C-30 in rat liver microsomes was studied by release of ^{3}H from [C-30-^{3}H]4, 4'-dimethylzymosterol (Fig. 1, II). A cyanide sensitive component of the system was purified to homogeneity and partially characterized.

EXPERIMENTAL

Materials. Rabbit and sheep antibodies to rat cytochrome b_5 were generous gifts from Dr. T. Omura and Dr. J. B. Schenkman, respetively. Goat antibody to rat cytochrome P-450 reductase was a generous gift of Dr. P. E. Thomas (Hoffmann-LaRoche, Inc.). Cytochrome P-450 reductase was prepared from calf liver (10), and a generous gift of rabbit liver enzyme was received from Dr. M. J. Coon. Cytochrome b_5 was prepared from rabbit liver (11), and steer liver enzyme was a generous gift from Dr. P. Strittmatter. All other enzymes and reagents were purchased from commercial sources and used without further purification. [^{3}H]Dimethylzymosterol was prepared from [^{3}H]mevalonolactone (manuscript in preparation). It was kept in benzene solution at 0-4°. Before use the solvent was removed and the substrate dissolved in dioxane(peroxide-free)-propylene glycol (2:1).

C-30 Oxygenase Assay. The reaction mixture (1 ml) contained 1 mM NADP, 2 mM $MgCl_2$, 5 mM GSH, 10 mM trisodium DL-isocitrate, 0.5 international units of porcine heart isocitrate dehydrogenase, [C-30-^{3}H]4, 4'-dimethylzymosterol (0.2 nmoles, approximately 500 μCi/μmole) and enzyme. After 30 min at 37°, 0.4 ml of 10% trichloroacetic acid was added, precipi-

tated proteins were removed by centrifugation, and the supernatant solution was extracted with 4 ml of chloroform on a Vortex mixer (cf. 12). The aqueous layer (0.7 ml) was stirred on a Vortex mixer with 0.3 ml of a suspension of 10% charcoal (Norit A), the charcoal was sedimented by centrifugation, and 0.5 ml of clear supernatant solution was counted with 10 ml of Aquassure (New England Nuclear).

Other Methods. Cytochrome b_5 was assayed according to Spatz and Strittmatter (11). Cytochrome P-450 reductase and cytochrome b_5 reductase were assayed with cytochrome C as electron acceptor according to Vermilion and Coon (13) and Mihara and Sato (14), respectively. Protein was determined according to Dulley and Grieve (15), and iron, according to Atkin et al (16). Sodium dodecylsulfate-polyacrylamide slab gel electrophoresis was done according to Laemmli (17) with 12% cross linked gels. The gels were stained with 0.25% Coomassie Blue R-250 in isopropanol acetic acid/water (25:10:65) overnight at 50°, and destained with isopropanol/acetic acid/water (10:10:80).

Purification of C-30 Oxygenase. Livers from male Sprague-Dawley rats (125-150 grams) were chilled on ice, washed with 0.25 M sucrose, homogenized in 2 volumes of 100 mM Tris-acetate buffer, pH 7.4, containing 30 mM NAM, 1 mM GSH, 10 mM EDTA and 150 mM KCl (Buffer A), and centrifuged at 10,000 x g for 20 min. All operations after liver removal were at 4°. The supernatant fraction was centrifuged at 105,000 x g for 90 min. The microsomal pellet was suspended in the same buffer without KCl and EDTA (Buffer B) and centrifuged at 105,000 x g. The washed microsomal pellet was suspended in Buffer B containing 20% glycerol (Buffer C), and treated with 10% Triton CF-10 and Buffer C (final concentrations of detergent and protein were 1% and 20 mg/ml, respectively). The suspension was stirred for 2.5 h, and centrifuged at 105,000 x g for 1.5 h. The supernatant fraction was treated with 50% polyethylene glycol-6000 (final concentration 5%), and centrifuged. The pellet was suspended in 5 mM KP_i buffer, pH 7.6, containing 30 mM NAM, 1 mM GSH, 0.1% Emulgen 913 (Kao-Atlas) and 20% glycerol (Buffer D), and the suspension was centrifuged at 130,000 x g for 3 h. The supernatant fraction was designated fraction A. (The pellet was discarded.)

The supernatant fraction from 0-5% polyethylene glycol was brought to 15% by addition of 50% polyethylene glycol and centrifuged. The pellet was dissolved in Buffer D (final volume 35 ml), and the solution was applied on a Sepharose 6-B column (2.6 x 84 cm) which was equilibrated with Buffer D. The column was developed with this buffer in 5 ml fractions which were assayed for C-30 oxygenase activity. Fraction A (100 μg protein) was added in this and subsequent assays. The active fractions (56-74) were pooled (90 ml) and applied on a DE-52 column (2.6 x 10 cm) equilibrated with 5 mM KP_i buffer, pH 8.0, containing 30 mM NAM, 1 mM GSH, 0.1% Emulgen 913 and 20% glycerol (Buffer E). The column was washed with Buffer E. The enzyme was not absorbed, and the effluent (120 ml) was applied on a hydroxylapatite (Calbiochem) column (2.6 x 10 cm) equilibrated with Buffer E. The column was washed with Buffer E, and active fractions of the effluent were pooled and concentrated by ultrafiltration with a PM-10 membrane (Amicon).

RESULTS AND DISCUSSION

The purification of C-30 oxygenase activity is summarized in Table I. NADH or NADPH were required and were equally effective as electron

Table I. Purification of C-30 Oxygenase

Fraction	Total activity	Specific activity[a]	Yield
	nmoles	nmoles/ mg protein	%
Microsomes	179	0.10	100%
Triton CF-10	160	0.18	89
Polyethylene glycol (5-15%)	143	0.41	80
Sepharose 6B	135	1.8	75
DE-52	119	4.0	66
Hydroxylapatite	56	37.6	31

[a] Determined at 0.2 μM 4, 4'-dimethylzymosterol, for which the apparent K_m is 2.0 μM.

donors at all stages of purification. The reaction was oxygen-dependent and CO-insensitive. Precipitation with polyethylene glycol resulted in 0-5% (Fraction A) and 5-15% fractions, neither of which was enzymically active. A combination of both in the assay procedure restored essentially complete activity. Addition of Fraction A in assays of all subsequent steps resulted in isolation of a homogeneous enzyme (C-30 oxygenase) with a polypeptide molecular weight of 29,000 (Fig. 2). As may be seen in Fig. 2 the enzyme stained weakly with Coomassie Blue. It was stable for 2 months at -18°. In presence of the 0.02% detergent in the assay phospholipids had no effect on enzyme activity.

Release of ^3H from substrate was 50% inhibited by 0.1 mM cyanide and it can be shown that the cyanide sensitive component is the purified enzyme (Table II). Both fraction A and the C-30 oxygenase (DE-52 step) were treated with 1 mM cyanide and passed through Sephadex G-25 columns. Incubation of treated fraction A with untreated oxygenase gave 70% of the activity of a control of untreated components, whereas nearly all activity was lost when the oxygenase was treated with cyanide. In agreement with

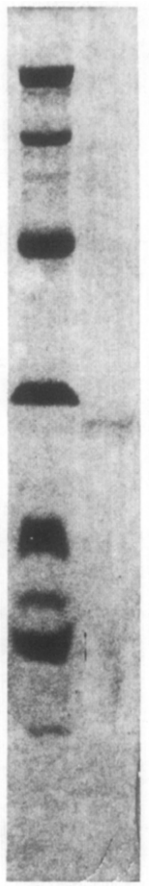

Fig. 2. Polyacrylamide slab gel electrophoresis of purified C-30 oxygenase (see text). Left lane, BioRad protein standards (5μg each): phosphorylase B 92,500, bovine serum albumin 66,200, ovalbumin 45,000, carbonic anhydrase 31,000, soybean trypsin inhibitor 21,500, and lysozyme 14,400. Right lane, 30 μg of purified oxygenase.

this interpretation was the finding that the purified C-30 oxygenase contained 1 gram-atom of non-heme Fe per 29,000 subunit. The product of the oxygenation reaction is under investigation. Previous work by Miller and Gaylor (18) with NAD-depleted microsomes indicated that the major oxygenated product was a 4α-carboxylic acid.

By anology with other mixed function oxidations one or more components of fraction A must function as redox proteins in the transfer of electrons from NADH or NADPH to the terminal oxygenase. The instability of fraction A has so far prevented us from purifying the other factor(s) required for C-30

Table II. Effect of cyanide on resolved C-30 oxygenase system.

Fraction treated with cyanide[a]	Activity	
	cpm/mg protein	% of control
None	4,880	100
Oxygenase	730	17
Fraction A	3,480	71
Oxygenase and fraction A	450	9

[a] Fraction A (4.1 mg protein) was treated with 1mM NaCN (final volume 1 ml) for 5 min at 4°. Free cyanide was removed by gel filtration on a Sephadex G-25 column (1.1 x 10 cm) equilibrated with buffer D. Oxygenase (from DE-52 step; 0.69 mg protein) was treated similarly. Assay procedure is described in text.

oxygenation. We have, therefore, attempted to implicate the known redox proteins of microsomal oxidations in C-30 oxygenation.

Goat antibody to rat cytochrome P-450 reductase did not inhibit C-30 oxygenation in microsomes, but inhibited cytochrome P-450 reductase by 75-90% of controls. Crowder and Brady obtained similar results in overall demethylation with ^{14}C-substrates (19). The concentration of NADH cytochrome b_5 reductase in fraction A is lower than 5% of that present in microsomes; NADPH cytochrome P-450 reductase is lower than 2%. Furthermore, thermal inactivation of these enzymes differs distinctly from that of the component active in C-30 oxygenation (Fig. 3).

Cytochrome b_5 was inactive in C-30 oxygenation when added to purified oxygenase, cytochrome P-450 reductase and NADPH, as it is under analogous conditions with stearyl CoA desaturase (20). Rabbit antibody to rat cytochrome b_5 from two different laboratories inhibited C-30 oxygenation in liver microsomes as reported previously (6, 7), but in our hands the control immunoglobulins were equally as inhibitory. Anti-

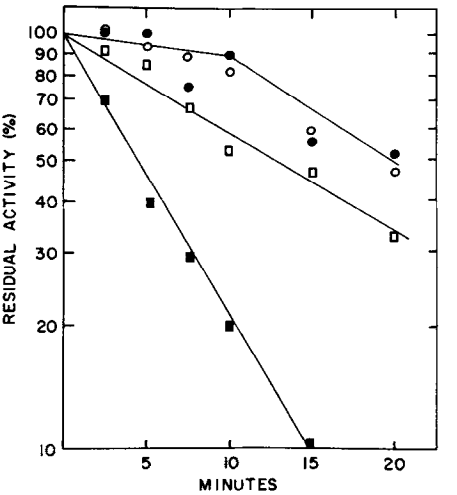

Fig. 3. Thermal stability of fraction A for C-30 oxygenation and for activity of cytochrome b_5 and cytochrome P-450 reductases. Fraction A was heated at 48°. Samples were withdrawn at times indicated, chilled, and assayed with purified C-30 oxygenase with NADH (●) and NADPH (○) as electron donors. Cytochrome b_5 reductase (■) and cytochrome P-450 reductase (□) were assayed as indicated in text.

cytochrome b_5 and control immunoglobulins were purified by chromatographic procedures (21). However, control immunoglobulin still inhibited C-30 oxygenation. On the other hand, NADH cytochrome b_5 reductase was inhibited by anti-cytochrome b_5 but not by control immunoglobulin. In trypsinized liver microsomes, the loss of cytochrome b_5 was paralleled by a loss in C-30 oxygenase activity which was restored by added cytochrome b_5 as reported previously for demethylase activity (7). However, the specificity of the reactivation may be questioned since cytochrome P-450 reductase, likewise removed by trypsin treatment, also restored activity. In accord with our observations is the finding by Buttke and Bloch (22) that the methyl groups at C-4 of lanosterol are removed in strain GL7, a heme and sterol deficient yeast mutant (8).

The non-heme iron enzyme acyl CoA desaturase has an absolute requirement for cytochrome b_5 as electron donor (23). Electron transfer to cytochrome b_5 from NADH or NADPH can occur by way of their specific

flavoprotein reductases, cytochrome b_5 reductase or cytochrome P-450 reductase, respectively (20). Our attempts to implicate these known redox proteins in electron transfer to the non-heme iron C-30 terminal oxygenase were not successful. Although their involvement is not completely excluded, it is suggested that other redox protein(s) participate in C-30 oxygenation. Further purification of fraction A should clarify the mechanism of electron transfer in this reaction.

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