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AFFINITY LABELING OF VERTEBRATE OXIDOSQUALENE CYCLASES WITH A TRITIATED SUICIDE SUBSTRATE

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Summary. Pig and rat liver oxidosqualene cyclase (OSC) enzymes were purified to homogeneity and showed single bands on SDS-polyacrylamide gel electrophoresis with molecular masses of 75 kDa (pig) and 78 kDa (rat). Pig liver OSC was purified for the first time (441-fold with a yield of 39%). Chemical affinity labeling of pure or crude preparations of the liver cyclases using the mechanism-based irreversible inhibitor of OSC, [^{3}H]29-methylidene-2,3-oxidosqualene ([^{3}H]29-MOS), showed a single radioactive band at 75 kDa (pig) and 78 kDa (rat). Affinity labeling experiments were also performed with dog and human microsomal preparations and with yeast and plant cyclases. All of the vertebrate OSC enzymes were specifically labeled with [^{3}H]29-MOS and gave a single band with molecular masses ranging from 70 to 80 kDa (rat, 78 kDa; dog, 73 kDa; pig, 75 kDa; and human, 73 kDa). In contrast, yeast lanosterol cyclase and plant cycloartenol cyclase were not labeled, demonstrating subtle differences in the active sites of animal, plant, and fungal enzymes.

The cyclization of 2,3-oxidosqualene to lanosterol is the most remarkable step in the biosynthesis of cholesterol. Oxidosqualene cyclase (OSC) (E.C. 5.4.99.7) binds (3*S*)-2,3-oxidosqualene folded into a chair-boat-chair conformation and then mediates sequential ring-forming reactions and backbone rearrangements through a series of rigidly-held carbocationic intermediates (1-7). However, the membrane-associated cyclases have been incompletely characterized because it was perceived that they were excessively difficult to purify as well as being unstable. Very recently, several OSCs were purified to homogeneity from vertebrate (8), yeast (9) and plant sources (10-12). The nature of the active site of these cyclases remains unknown.

We recently reported the first potent mechanism-based irreversible inactivator of lanosterol cyclase, 29-methylidene-2,3-oxidosqualene (29-MOS), which made it possible to stoichiometrically and covalently modify the active site of the enzyme (13). The mechanism of

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hhibition appears to involve initial cyclization of 29-MOS to the allylic 21-methylidene-
lrotosterol C-20 cation, which can be trapped by an active-site nucleophile, resulting in
lreversible inhibition of the cyclase (Scheme 1) (13). We describe herein the chemical affinity
abeling of the two homogeneous vertebrate liver OSCs (rat and pig) using tritium-labeled
l9-MOS. In addition, labeling experiments with [^{3}H]29-MOS were carried out using
hicrosomal preparations of dog liver, human liver and human Hep G2 cells; yeast and pea
leedling cyclase activities were also examined. This is the first direct species comparison for
he vertebrate OSC enzymes that employs an active-site directed affinity label.

MATERIALS AND METHODS

hemicals. [^{3}H]29-Methylidene-2,3-oxidosqualene ([^{3}H]29-MOS, 2.3 Ci/mmol) and
S)-[24,30-^{14}C]2,3-oxidosqualene (52 mCi/mmol) were synthesized as described (13,14).
EAE-Sephacel and Blue Sepharose 4B were purchased from Pharmacia, hydroxylapatite
Bio-Gel HTP) from Bio-Rad, and Triton X-100 from Sigma.

iological Materials. Human liver was obtained from the Liver Tissue Procurement and
istribution System (H. Sharp, M.D., University of Minnesota). Microsomal preparation of
uman hepatoma G2 cell (16 mg protein/ml) and dog liver (10 mg protein/ml), both from
luvastatin(R)-induced tissues, were generous gifts from Dr. B. Boettcher (Sandoz Research
stitute). Microsomal preparations of pea seedlings and solubilized baker's yeast cyclase
ere prepared as described (10,15).

nalytical Methods. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed
ith 7.5% or 15% acrylamide gels (16). Proteins were visualized by Coomassie Brilliant Blue
aining. The molecular masses for the purified enzymes were estimated using protein
tandards (Bio-Rad). Protein concentrations were determined by a modified Lowry method
7), with bovine serum albumin as standard. N-Terminal amino acid sequencing by
e Edman method was carried out using an Applied Biosystems Model 475A pulsed liquid
hase sequencer, and the amino acid composition analysis was accomplished using a
Vaters/Millipore Picotag System following vapor phase hydrolysis (6 N HCl, 110 °C for 24 h)
y T. Fischer at the Center of Analysis and Synthesis of Macromolecules (Stony Brook).

urification of Oxidosqualene Cyclase. All manipulations for enzyme preparation were
arried out at 4 °C. Frozen pig liver (50 g) was homogenized in a Waring blender in
e presence of 2 volumes of 0.1 M Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM DTT,
0 µg/ml phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 2.5 µg/ml aprotinin, and
e microsomal fraction was prepared as described (18). The microsomes were suspended
150 ml of 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM DTT, and 0.5% (w/v)
riton X-100. The mixture was gently stirred for 60 min and centrifuged at 105,000 x *g* for
0 min and the cyclase was obtained in the supernatant.

he solubilized pig liver cyclase was applied to a DEAE-Sephacel column (2 x 15 cm) equilibrated
ith 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1mM DTT, and 0.5% Triton X-100 (buffer A)
t a flow rate of 60 ml/h. After washing the column with 75 mM KCl in buffer A, the cyclase activity
as eluted with 100 mM KCl in buffer A. After concentration and dialysis, the DEAE purified
yclase was then loaded on a hydroxylapatite column (1.5 x 15 cm) equilibrated with 5 mM
otassium phosphate buffer (pH 7.4) containing 1 mM DTT and 0.2% Triton X-100 at a flow rate of
0 ml/h, and the cyclase activity was recovered in a non-adsorbed fraction. The purified pig liver
SC (1.2 mg) showed a single band with molecular mass of 75 kDa by SDS-PAGE.

Rat liver cyclase was purified to homogeneity in a similar way, except that squalene epoxidase (SE) was removed by adsorption of the DEAE eluate to Blue Sepharose 4B. Cyclase activity was eluted with 50 mM KCl in buffer A, while SE activity was adsorbed to the Blue Sepharose (18). The DEAE/Blue Sephacel-purified cyclase was loaded onto a hydroxylapatite column as described above. The cyclase activity was quantitatively recovered in non-adsorbed fraction, and the purified rat liver OSC showed a single band with molecular mass of 78 kDa on SDS-PAGE.

Enzyme Assay. An aliquot of enzyme solution in a total volume of 240 μl (final concentration or Triton X-100, 0.1%) was pre-incubated at 37 °C for 10 min. Then, [^{14}C](3*S*)-2,3-oxidosqualene (20 μM, 20,000 cpm) in 2 μl of 2-propanol was added into the mixture and incubated at 37 °C for 50 min. The enzymatic reaction was quenched by addition of 240 μl of 10% KOH in methanol, and further incubated for 1 h. The nonsaponifiable lipids were extracted with 1 ml of methylene chloride and the organic extracts were evaporated in a Savant SpeedVac. The residue was ther dissolved in 100 μl of methylene chloride, applied to pre-channeled silica gel TLC plates (Whatman silica gel 60A), and developed with 10% ethyl acetate - hexane. The enzyme activities were analyzed by radio-TLC scanning using an Imaging Scanner (Bio-Scan, System 500). R_f values were 0.56 for oxidosqualene and 0.23 for lanosterol.

Chemical Affinity Labeling. Crude or purified OSCs (20-100 μg of protein in a total volume of 240 μl) were incubated with [^{3}H]29-MOS (1.0 μM, 2.6 x 10^5 cpm) in the presence of 0.1% Triton X-100 for 15 min at 37 °C. For yeast cyclase, incubation was carried out in the presence of 1.4% Triton X-100 as described before (15). The affinity-labeled proteins were precipitated with 50% acetone, lyophilized, dissolved in SDS sample buffer, and separated by 7.5% SDS-PAGE. The gel was stained, enhanced with 15% 2,5-diphenyloxazole, shrunken with 50% aqueous PEG 2000 (19), dried and exposed to a pre-flashed Kodak X-OMAT XAR-5 film at -80 °C for 3 days. For yeast and pea seedling cyclase, the incubations were performed at 30 °C overnight; under these conditions, both enzyme preparations showed more than 90% conversion of oxidosqualene (20 μM) into lanosterol. The samples were then applied to SDS-PAGE (15%), and exposed to the film for one week or longer.

Results and Discussion

The oxidosqualene cyclases are thought to be unstable, membrane-bound enzymes and difficult to purify. Recent reports on purification of OSCs have required numerous steps and the yield of purified protein was rather low (8-12). However, we found that vertebrate (rat and pig) liver OSCs can be conveniently purified to homogeneity by modifying the reported purification methods (8,20), and employing a simple combination of DEAE-Sephacel and hydroxylapatite column chromatographies after solubilization with Triton X-100. Both pig and rat liver cyclases could be purified similarly. Solubilized liver OSC was eluted from a DEAE-Sephacel column and applied to a hydroxylapatite column. OSC activity was recovered in the non-adsorbed fraction, and this material showed a single band on SDS-PAGE with molecular mass of 75 kDa (pig) and 78 kDa (rat). The reported value of molecular mass for rat liver OSC is 75 kDa (8), which is consistent with our results. For the first time, pig liver OSC was completely purified up to 441-fold with a yield of 39% (Table 1). We scaled up this purification method and obtained 33 mg of pure pig OSC from 4 kg of liver.

For both liver OSCs, N-terminal amino acid sequencing by the Edman method was attempted; however, the N-terminus was found to be blocked as previously reported for yeast lanosterol cyclase (9). The approximate amino acid composition for pig liver cyclase was

Table 1. Purification of (3*S*)-2,3-oxidosqualene:lanosterol cyclase from pig liver

	Protein (mg)	Total Activity (nmol/h)	Specific Activity (nmol/h/mg)	Yield (%)	Fold
Microsomes	1350	8100	6	100	1
Solubilized	347	8111	23	100	4
DEAE-Sephacel	51.4	4387	85	54	14
Hydroxylapatite	1.2	3171	2643	39	441

determined as follows (excluding Cys and Trp, which were not determined): Asx (55.5), Glx (81), Ser (43), Gly (54), His (23), Arg (49), Thr (37), Ala (48), Pro (37), Tyr (37), Val (30.5), Met (4), Ile (19), Leu (67), Phe (23), Lys (13).

Chemical affinity labeling experiments using 29-MOS with high specific radioactivity were performed with both pure and crude preparations of the liver cyclases. After incubation with [^{3}H]29-MOS (1.0 μM, 2.3 Ci/mmol) for 15 min at 37 °C, each sample was applied to SDS-PAGE and the covalently-modified protein band was visualized by fluorography. In both cases, a single radioactive band was observed on the fluorogram at the same molecular mass as described above, 75 kDa (pig) and 78 kDa (rat), which clearly confirmed that these protein bands are responsible for the cyclase activity (Figure 1).

In order to compare and contrast OSC enzymes obtained from different taxa, the labeling experiments were also performed with crude microsomal preparations of dog liver, human liver, human hepatoma G2 cells, yeast, and pea seedlings. The pea enzyme catalyzes the cyclization of 2,3-oxidosqualene into cycloartenol, a biosynthetic precursor of plant sterols; the postulated mechanism is essentially the same as that for lanosterol cyclization, except for the final cyclopropane ring closure instead of H-9 proton elimination (10). Each of the vertebrate lanosterol cyclases was specifically labeled with [^{3}H]29-MOS within minutes and showed a single radiolabeled protein band with molecular mass of 70 - 80 kDa range on the fluorogram: rat, 78 kDa; dog, 73 kDa; pig, 75 kDa; human and hep G2, 73 kDa (Figure 2).

In contrast, the yeast and pea cyclases were not labeled even under forcing conditions. For these two preparations, incubations were carried out overnight, and the gel was exposed

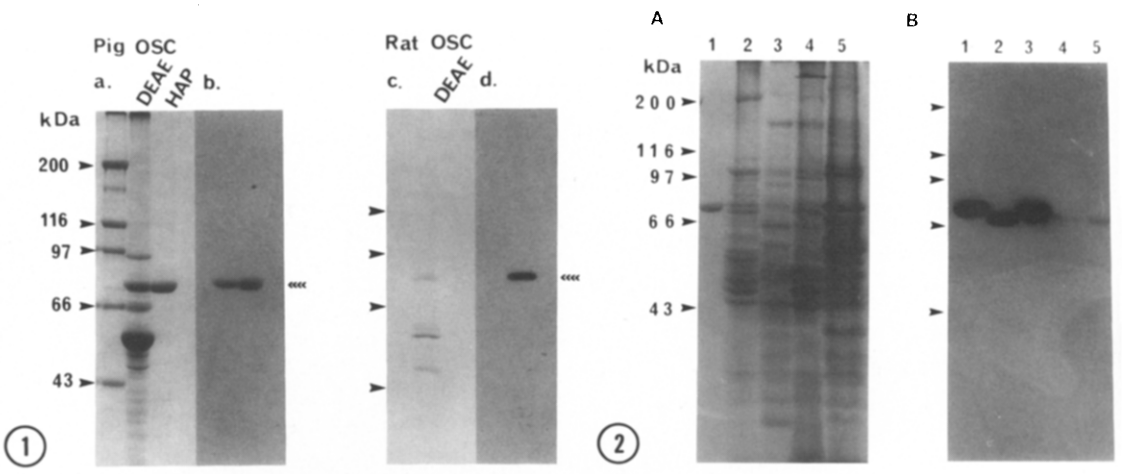
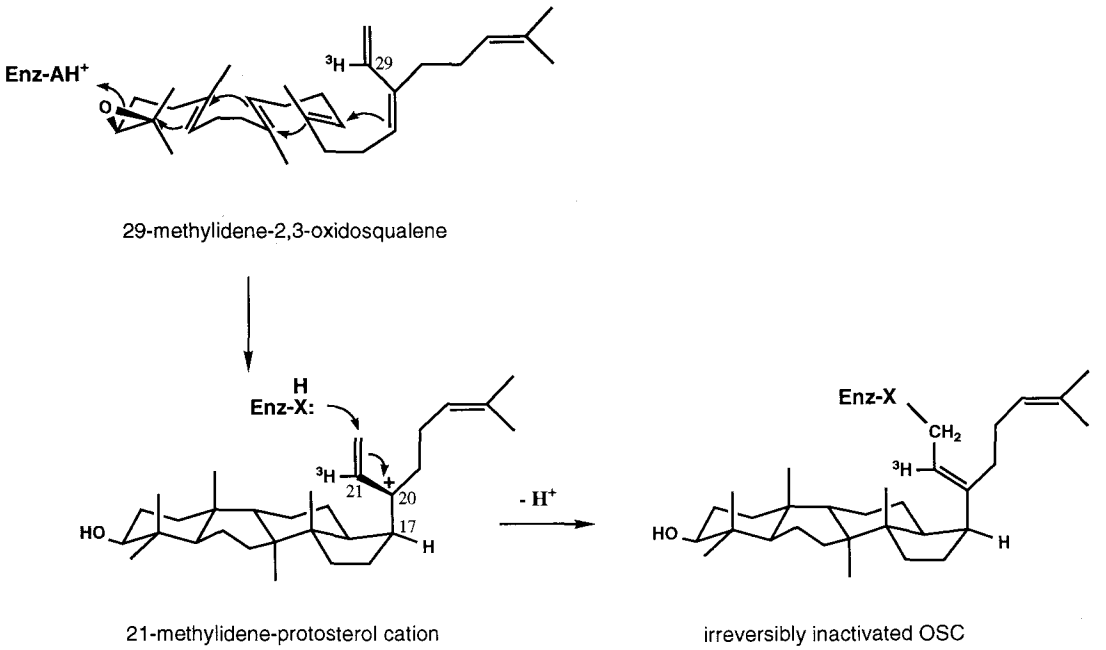


Figure 1. Chemical affinity labeling of crude and purified pig liver OSC with [^{3}H]29-MOS. (**a**) and (**c**) SDS-PAGE (7.5%) gel stained with Coomassie Brilliant Blue; (**b**) and (**d**) corresponding fluorogram. Lane **1**, DEAE partially-purified cyclase; lane **2**, purified cyclase.

Figure 2. Chemical affinity labeling of OSCs from various species. (**A**) SDS-PAGE (7.5%) gel stained with Coomassie Blue; (**B**) corresponding fluorogram. Lane **1**, purified rat liver OSC; lane **2**, dog liver microsomes; lane **3**, pig liver microsomes; lane **4**, human liver microsomes; lane **5**, human hepatoma G2 cell microsomes. Equal OSC activity was loaded in each lane.

to X-ray film for a much longer period. Control enzyme preparations showed more than 90% conversion of 20 μM of [^{14}C]-oxidosqualene. Furthermore, it was confirmed that when either of these two incubation mixtures was loaded on a DEAE-Sephacel column equilibrated with 10 mM phosphate buffer (pH 7.0), essentially all of the radioactivity was recovered in non-adsorbed fraction, while cyclase activity was eluted with 200 mM phosphate. (For the irreversibly-labeled vertebrate cyclases, radioactivity co-elutes with the inactivated cyclase protein (13).) For the yeast cyclase reactions, most of 29-MOS was recovered unchanged on TLC, with little conversion to lanosterol-like products. The active site structure of these cyclases apparently differs from those of vertebrate cyclases, at least in the position of a key nucleophilic residue. Indeed, the reported molecular mass of the purified yeast cyclase is 26 kDa (9) and that of pea cycloartenol cyclase is 55 kDa (10); these values differ considerably from the 70 - 80 kDa vertebrate OSCs.

The proposed mechanism of inhibition of OSC by 29-MOS involves initial cyclization to 21-methylidene-protosterol cation, followed by trapping of this allylic cation by an active-site nucleophile and concomitant irreversible inactivation of the cyclase (Scheme 1). Corey and Virgil have proposed that the protosterol cation should have a 17β-oriented side chain instead of 17α-arrangement as previously suggested (21,22,23). The active-site nucleophile that is trapped could be one of the residues that normally stabilizes the C-20 protosterol cation and assures the following backbone rearrangement to yield lanosterol. For pig liver cyclase, an IC_{50} value of 0.5 μM, an apparent K_I value of 4.4 μM, a k_{inact} value of 221 min^{-1}, and a partition



Scheme 1. Proposed mechanism of irreversible inhibition of OSC by 29-MOS.

ratio of 3.8 were reported (13). Incubation of 0.1 μM 29-MOS with pig liver OSC gave a polycyclic product in 30% yield, although at [29-MOS] > K_I, complete inactivation precluded isolation of product (13). Failure of labeling of yeast and plant cyclase might be caused simply by the absence of the active-site nucleophilic residue that traps the 21-methylidene protosterol cation in the vertebrate OSC enzymes.

Since 29-hydroxy-2,3-oxidosqualene is quantitatively converted to 21-hydroxylanosterol by yeast cyclase (15), it seems most likely that 29-MOS is also a substrate for the yeast OSC. The initially formed methylidene protosterol cation could be stabilized by the enzyme residue so that the backbone rearrangement does not further proceed to yield the lanosterol analogue. Further, C(20) of the cyclic cation should be shielded from water molecules, since (20*E*)-20,21-dehydro-2,3-oxidosqualene is converted to (17*S*,20*S*)-protostan-24-ene-3β,20-diol by yeast OSC (22). In this case, the stabilization is not attained by tight covalent bonding like vertebrate cyclase, but probably attained by an electrostatic interaction, and as a result 29-MOS appears to act as a slow tight-binding inhibitor with an IC_{50} value 1.5 μM (13), an apparent K_I value of 1.5 μM, and a k_{inact} value of 0.088 min^{-1}. Similarly, the pea seedling microsomal OSC showed an IC_{50} value of 2 μM for 29-MOS. Very recently, 10,15-didesmethyl-2,3-oxidosqualene was found to be a time-dependent irreversible inhibitor of yeast OSC, while it is converted to a cyclic product by crude pig liver OSC (24). The different behavior of the vertebrate, yeast, and pea cyclases towards suicide substrates, as well as the considerable difference in their molecular sizes, suggests species differences in the structures of the active site as well as global differences.

In conclusion, [^{3}H]29-MOS has been shown to be a specific substrate for stoichiometric, covalent modification of the active site of vertebrate OSCs. Identification of the covalently-modified residues, mapping of active-site peptides, and cDNA cloning of the purified vertebrate liver cyclases is now in progress.

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