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Purification, tandem mass characterization, and inhibition studies of oxidosqualene-lanosterol cyclase enzyme from bovine liver

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Abstract

The oxidosqualene-lanosterol cyclase (OSC) from bovine liver has been isolated from the microsomal membrane fraction and purified to homogeneity by ultracentrifugation, Q-Sepharose, hydroxyapatite, and HiTrap heparin chromatographies. The purified protein required Triton X-100 to retain its highest activity. The cyclase had a molecular mass of ~70 and ~140 kDa, as evidenced by a single protein band on silver-stained SDS–PAGE and Coomassie-stained PAGE, respectively. Results from Edman degradation of OSC suggested that it might have a blocked N-terminus. Further peptide mapping coupled with tandem mass spectrometric determination identified three peptide fragments, ILGVGPDDPDLVR, LSAEEGPLVQSLR, and NPDGGFATYETK, which are highly homologous to human, rat, and mouse OSCs. The purified cyclase showed pH and temperature optima at pH 7.4 and 37 °C, respectively. The apparent K_M and k_{cat}/K_M values were estimated to be 11 μM and 1.45 mM^{-1} min^{-1}, respectively. Inhibition studies using both Ro48-8071 and *N*-(4-methylenebenzophenonyl)pyridinium bromide showed potent inhibition of OSC with an IC_{50} of 11 nM and 0.79 μM, respectively. Results from DTNB modification and DTNB coupled with Ro48-8071 competition study suggest that two sulfhydryl groups are involved in the catalysis but not located in the substrate binding pocket or catalytic active site. The purified OSC was maximally inactivated by diethyl pyrocarbonate near neutral pH and re-activated by hydroxylamine, indicating the modification of histidine residues. The stoichiometry of histidine modification and the extent of inactivation showed that two essential histidine residues per active site are necessary for complete bovine liver OSC activity.

Keywords: Oxidosqualene cyclase; Ergosterol biosynthesis; 5,5′-Dithio-bis-(2-nitrobenzoic) acid; Diethyl pyrocarbonate

Studies of (oxido)squalene cyclases (EC 5.4.99.7) have intrigued scientists for many decades, with specific interest in the complex cyclization/rearrangement mechanisms they catalyze, and their potential applications in the design of antifungal, hypocholesterolemic, and phytotoxic chemotherapeutics [1–3]. In the conversion of (3*S*)-2,3-oxidosqualene to lanosterol or cycloartenol, at least 10 formal covalent changes including cyclization and rearrangement reactions are involved to form tetracyclic and pentacyclic triterpenoids in a single biosynthetic step (Fig. 1). In parallel, the prokaryotic squalene-hopene cyclase (SHC)[2] catalyzes the cyclization of squalene to pentacyclic triterpenoids, acting as a structural and functional equivalent of eukaryotic sterols, without hydride and methyl group rearrangement. Potent inhibitors of oxidosqualene cyclase enzymes, designed to mimic non- or partially cyclized transition states and high-energy intermediates, have been synthesized, and the antifungal and cholesterol-lowering effects of these inhibitors have been demonstrated [4–6].

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[2] *Abbreviations used:* OSC, oxidosqualene-lanosterol cyclase; CAS, oxidosqualene-cycloartenol synthase; SHC, squalene-hopene cyclase; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic) acid; DEPC, diethyl pyrocarbonate. Enzymes: oxidosqualene-lanosterol cyclase (EC 5.4.99.7); oxidosqualene-cycloartenol synthase (EC 5.4.99.8).

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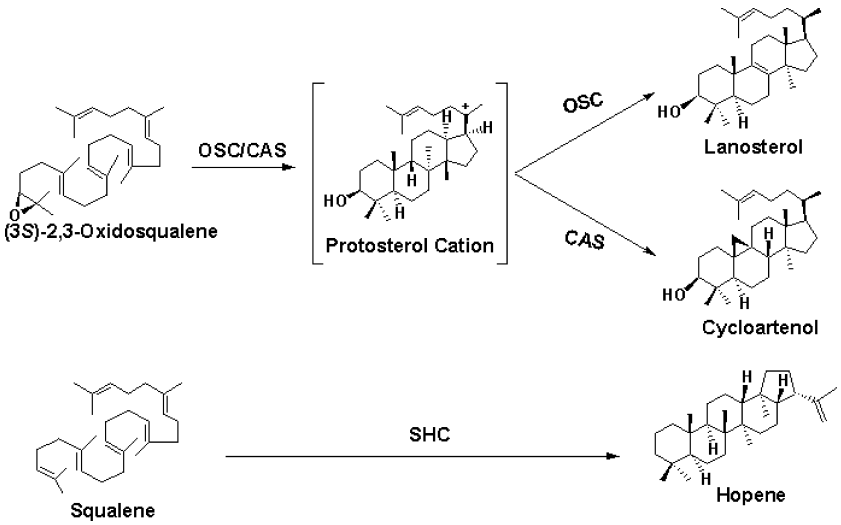

Fig. 1. Enzymatic reactions catalyzed by (oxido)squalene cyclases. Squalene-hopene cyclase (SHC) catalyzes the conversion of linear squalene to hopene. Both oxidosqualene-lanosterol cyclase (OSC) and oxidosqualene-cycloartenol synthase (CAS) transform the common substrate, (3*S*)-2,3-oxidosqualene, to lanosterol and cycloartenol, respectively, via a common tetracyclic protosterol cation intermediate.

Both chemical and molecular biological studies have significantly contributed mechanistic insights into the (oxido)squalene cyclase-catalyzed cyclization/rearrangement cascade. For example, cloning, sequencing, and site-directed mutational studies of putative (oxido)squalene cyclase genes have facilitated understanding of cyclase evolution and analysis of factors that determine product diversity, perhaps the single most remarkable feature of cyclase enzymes [2,7–16]. Additionally, crystallization and structural characterization of SHC from *Alicyclobacillus acidocaldarius* have provided a detailed model for determination of the substrate entrance channel and catalytically important active-site residues [17,18]. Recently, we used random mutagenesis coupled with plasmid shuffle strategies to simultaneously identify five unique point mutations that alter product specificity. These mutations were responsible for changing cyclase from oxidosqualene-cycloartenol synthase (CAS) to oxidosqualene-lanosterol cyclase (OSC) activity. In addition, we were able to estimate the frequency with which such mutations arose [16].

Although structural characterization of active-site residues on SHC-catalyzed cyclization is available, details of X-ray structural information on oxidosqualene cyclase are still lacking, making the in-depth characterization of substrate pre-folding and cation stabilization as well as product specificity of oxidosqualene cyclase-catalyzed cyclization/rearrangement reactions unfeasible [17–19]. Besides, comparison of amino acid sequences among different eukaryotic oxidosqualene cyclases and prokaryotic squalene cyclase showed only moderate identity, even though several highly conserved motifs rich in aromatic amino acids were identified and the overall 3-D structures between both enzymes are highly homologous [16,20,21]. These results suggest that the cyclase enzymes may maintain the conserved general active-site structure but develop diverse product specificities via subtle changes of the shape of the active site and/or the position of the crucial functional groups throughout the course of evolution. To understand the structure–function relationships of oxidosqualene cyclase-catalyzed reactions, the purification and biochemical characterization of oxidosqualene cyclase enzyme are prerequisite.

Purification of oxidosqualene cyclases has been hampered until recently, although a few oxidosqualene cyclases have been purified to homogeneity, largely due to the considerable challenges of purification from microsomal fractions [22–26]. Because understanding the mechanism of the OSC-catalyzed cyclization/rearrangement reaction is of fundamental importance, we report herein the purification, identification, and biochemical characterization of OSC from bovine liver, as well as inhibition studies. Specifically, in this paper we demonstrate (a) detergent dependence of the enzymatic activity, (b) succinct purification of OSC using three types of chromatography, (c) identification of the purified enzyme using tandem mass spectrometry, (d) pH and temperature optima for the enzyme, (e) inhibitory kinetics of mechanism-based inhibitors on the purified enzyme, and (f) involvement of both cysteine and histidine residues in the enzyme catalysis.

Materials and methods

Materials

All chemical reagents were purchased from Sigma unless specified otherwise. Protein molecular weight

standards, hydroxyapatite resin, and detergent compatible protein assay kit were obtained from Bio-Rad. Q-Sepharose Fast Flow and HiTrap heparin column were provided by Amersham–Pharmacia Biotech. Fresh male bovine liver was purchased from local supply in Taiwan. [4,8,12,13,17,21-^{3}H]Squalene, [1,5,9,14,20,24-^{14}C]squalene, and [4,8,12,13,17,21-^{3}H]oxidosqualene were from American Radiolabeled Chemicals. [1,5,9,14,20,24-^{14}C]Oxidosqualene (37×10^6 dpm/μmol) was synthesized as reported [27].

Microsomal membrane preparation and purification of oxidosqualene-lanosterol cyclase

Homogenization and all subsequent isolation and purification procedures were performed at 4 °C. Fresh male bovine livers (300 g) were blended at 4 °C in HB I buffer (600 mL), Tris buffer (100 mM, pH 7.4) containing 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, and 40 μg/mL phenylmethylsulfonyl fluoride (PMSF) and centrifuged for 30 min at 10,000*g*, cellular debris was discarded, and the supernatant was centrifuged at 123,000*g* for 1 h. The microsomes were re-suspended in HB I buffer (200 mL) and re-centrifuged for 1 h at 123,000*g*. Washed microsomes were suspended in HB II buffer (100 mL), Tris buffer (20 mM, pH 7.4) containing 1 mM EDTA, 1 mM benzamidine, 40 μg/mL PMSF, and 1 mM DTT, supplemented with 0.5% Triton X-100 and gently stirred for 1 h. Crude enzyme extract was separated from the microsomal membrane fraction by centrifugation at 123,000*g* for 1 h and re-suspended in HB II buffer containing 0.5% Triton X-100 to a concentration of 4 mg/mL.

The crude enzyme extract was first applied on a Q-Sepharose Fast Flow column (2.5×20 cm) equilibrated with IEB buffer (HB II buffer + 0.5% Triton X-100). The column was washed with 1 L IEB buffer and then eluted with 5 void volumes of IEB buffer containing 20, 50, and 100 mM KCl. The active fractions were pooled, dialyzed against HAB buffer, potassium phosphate buffer (5 mM, pH 7.4) containing 1 mM DTT and 0.5% Triton X-100, and concentrated using YM30 filter membrane. The cyclase-containing sample was loaded onto the hydroxyapatite column (2.5×10.5 cm) equilibrated with the same buffer. The column was washed with 400 mL HAB buffer. Since OSC was not adsorbed on the gel, fractions of 10 mL of flowthrough were collected and assayed for cyclase activity. The active fractions were next applied on HiTrap heparin columns (4×5 mL) equilibrated with HB buffer, 5 mM potassium phosphate buffer (pH 7.4) containing 0.5% Triton X-100, and then eluted with 3.5 volumes of stepped 50 mM, 100 mM, and 1 M KCl. Enzyme was eluted between 50 and 100 mM KCl; fractions of 2 mL were collected and portions (200 μL) of each fraction were assayed for OSC activity. Protein concentrations were determined by the method of modified Lowry assay, with bovine serum albumin as the standard [28]. Protein samples that contained detergent but not reducing agents were directly assayed with detergent compatible protein assay kit, according to supplier's instructions. Polyacrylamide gel electrophoresis was carried out by the method of Laemmli and visualized with silver staining [29].

Oxidosqualene-lanosterol cyclase activity assay and biochemical property characterization

Enzyme activity was determined by a slight modification of published procedures [16,27]. The reaction mixture contained 5 mM phosphate buffer (pH 7.4), 0.5% Triton X-100, and 50 μM [*R*,*S*]2,3-oxidosqualene or [^{3}H][*R*,*S*]2,3-oxidosqualene (3×10^6 dpm/μmol) in a final volume of 200 μL. Cyclase-containing protein (0.025 mg) was added to initiate the reaction at 37 °C for 2 h with shaking. Following the termination of reaction with addition of equal volume of 15% ethanolic KOH, the sample was extracted twice with an equal volume of CH_2Cl_2. The pooled organic layers were evaporated to dryness and re-dissolved in 50 μL CH_2Cl_2. The extract was applied onto a silica gel thin-layer chromatography (TLC) plate (Merck No. 5714-3), developed with hexane/ethyl acetate (4:1, v/v), and visualized with sprayed anisaldehyde reagent followed by heat incubation or directly scanned with Bio-Scanner instrument.

For determination of K_M and V_{max} values, substrate concentrations of 2–500 μM and a suitable time point which shows linear relationship between the concentration of lanosterol as a function of time were carried out. The data collected were next applied to the Lineweaver–Burk equation. The temperature stability of the enzyme was determined by incubation of the reaction mixture at various temperatures (25, 37, 50, 60, and 70 °C) for 30 min before residual activity was measured. Determination of the optimal pH profile was carried out in a final volume of 200 μL at various pHs, using the following buffers (5 mM): citrate (pH 4.0, 5.0, and 6.0), Tris–HCl (pH 6.0, 7.0, 7.4, 8.0, and 9.0), potassium phosphate (pH 6.0, 7.0, 7.4, 8.0, and 9.0), and CAPS (pH 9.0 and 10.0), 500 μM OS, 0.5% Triton X-100, and 0.025 mg of purified enzyme as described above.

Peptide sequencing by tandem mass spectrometry

The OS cyclase-containing fractions obtained from the HiTrap heparin column were collected, concentrated, and desalted by using a Centriprep concentrator and then subjected to SDS–PAGE. Following the electrophoresis, the gel was stained with Coomassie blue. The OSC band was excised and in-gel digested with

trypsin in NH_4HCO_3 solution (25 mM, pH 8.0) [30]. A small aliquot of the generated peptide mixture was analyzed by nanoflow capillary liquid chromatography tandem mass spectrometry.

The column of nanoscale capillary liquid chromatography (Ultimate, LC Packings, Amsterdam, Netherlands) used was a reversed-phase C18 (15 cm × 75 μm i.d., 3 μm) with a flow rate of 200 nL/min. The tryptic peptides were separated by a 30 min gradient of 5–50% solvent B (A = 2% acetonitrile/0.1% formic acid and B = 98% acetonitrile/0.08% formic acid). The eluted peptides were then introduced into a Q-TOF mass spectrometer (QSTAR Pulsar *i*, Applied Biosystems, Foster City, CA, USA). Tandem mass spectra were automatically collected under the Information-Dependent Acquisition (IDA) during the 60 min LC/MS/MS run. Peptide product ion spectra generated by LC/MS/MS were searched against the SWISS-PROT protein databases using the Mascot search program sequence database search engine [31]. The MS BLAST was then carried out for searching consensus protein sequence composed of aligned sequences from database using peptide sequences produced by mass spectrometry [32].

N-terminal amino acid sequencing

The N-terminal amino acid sequences were determined using an Applied Biosystems Procise Protein Sequencing System that performs hydrolysis, derivatization, and HPLC of the phenylthiohydantoin-amino acid automatically, according to supplier's instructions. A thiol reductant was utilized as a scavenger to protect residues from oxidation during acid hydrolysis. The sequencing analysis of N-terminus of OSC was performed on material blotted from SDS–PAGE gel onto a PVDF membrane by the method of Matsudaira [33].

Synthesis of putative OSC inhibitors and inhibition studies of OSC

Putative OSC inhibitor, Ro48-8071, was synthesized as reported and the spectroscopic data were identical to those published in the literature [6]. For the synthesis of *N*-(4-methylenebenzophenonyl)pyridinium bromide, a 5 mL pyridine solution containing 4-(bromomethyl)benzophenone (100 mg, 0.36 mmol) was refluxed with stirring under nitrogen for 1 h. The *N*-(4-methylenebenzophenonyl)pyridinium bromide (85 mg, 0.24 mmol, 67%) was obtained as a brown solid after removal of excess pyridine and twice lipophilic Sephadex column purification (CH_2Cl_2:MeOH 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.51 (s, 2H), 7.43–7.47 (m, 2H), 7.56–7.60 (m, 1H), 7.70–7.74 (m, 4H), 7.91–7.92 (m, 2H), 8.10 (m, 2H), 8.51 (m, 1H), 9.77 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) d 63.41, 128.67, 129.80, 130.14, 130.22, 131.08, 133.19, 136.85, 137.31, 139.02, 145.51, 145.82, 196.01; FAB MS for $C_{19}H_{16}ON^+$ (M^+) 274.124100 (calc. 274.123189). For the synthesis of *N*-(3-methylenebenzophenonyl)pyridinium bromide, a 3 mL pyridine solution containing 3-(bromomethyl)benzophenone (50 mg, 0.18 mmol) was refluxed with stirring under nitrogen for 2 h. Excess pyridine was removed at reduced pressure affording a brown semisolid. The *N*-(3-methylenebenzophenonyl)pyridinium bromide (56 mg, 0.16 mmol, 91%) was obtained as a brown solid after lipophilic Sephadex column purification (CH_2Cl_2:MeOH 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.53 (s, 2H), 7.43–7.71 (m, 7H), 8.07–8.12 (m, 4H), 8.49–8.53 (m, 1H), 9.73–9.75 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) d 63.61, 128.71, 128.76, 129.80, 130.27, 130.39, 131.65, 133.22, 133.84, 134.10, 136.86, 138.85, 145.47, 145.86, 195.92; FAB MS for $C_{19}H_{16}ON^+$ (M^+) 274.124100 (calc. 274.123189). For the synthesis of 2-(bromomethyl)benzophenone, a solution of 2-methylbenzophenone (200 mg, 1.02 mmol) in CCl_4 was added with 1.5 eq. NBS (272 mg, 1.53 mmol) and AIBN (2%, 3 mg, 0.02 mol). The mixture was refluxed with stirring under argon for 4 h. Succinimide was filtered off at room temperature and washed once with CCl_4. Solvent was removed from the filtrate at reduced pressure affording yellowish oil. Starting material and 2-(bromomethyl)benzophenone were separated from impurities on a silica gel column (hexane:ethyl acetate 5:1). The mixture was dissolved in excess dry pyridine (10 mL) and refluxed with stirring under argon overnight. Excess pyridine was removed at reduced pressure affording brown oil. The *N*-(2-methylenebenzophenonyl)pyridinium bromide (50 mg, 0.14 mmol, 14% for 2 steps) was obtained as a yellow solid after lipophilic Sephadex column purification (CH_2Cl_2:MeOH 1:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.30 (s, 2H), 7.47–7.54 (m, 4H), 7.63–7.74 (m, 4H), 8.07–8.11 (t, 2H), 8.22–8.24 (d, 1H), 8.53–8.55 (m, 1H), 9.62–9.63 (d, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) d 61.18, 127.85, 128.56, 129.43, 130.40, 130.86, 131.75, 132.71, 133.39, 133.88, 136.58, 137.66, 145.13, 145.42, 197.57; FAB MS for $C_{19}H_{16}ON^+$ (M^+) 274.124100 (calc. 274.123189).

Inhibition assays were performed by mixing the (3*S*)-OS/[^{3}H](3*S*)-OS (50 μM, 3×10^6 dpm/μmol) with purified bovine liver OSC and different concentrations of the inhibitor in 2.5% DMSO. The control experiment contained no inhibitor, but 2.5% DMSO instead. Purified OSC (0.025 mg, 50 μM, 20 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, and 0.5% Triton X-100) was incubated with (3*S*)-OS/[^{3}H](3*S*)-OS and various concentrations of inhibitors for 2 h with shaking at 37 °C. The reaction was quenched with 15% ethanolic KOH, incubated at 65 °C for 15 min, and then extracted as described above. IC_{50} value for the inhibitor was determined by plotting % of OSC activity versus log inhibitor concentration.

Chemical modification of OSC with 5,5′-dithio-bis-(2-nitrobenzoic) acid

A stock solution containing 10 mM 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) was prepared in methanol. The reaction mixture (final volume, 1 mL) containing 0.5% Triton X-100, 120 μL of purified OSC (~0.5 mg/mL), and various concentrations (0, 20, 40, 60, 80, and 100 μM) of DTNB was incubated at 37 °C. Quantification of reactive sulfhydryl group was carried out by monitoring the time-dependent change in absorbance at 412 nm, which corresponds to the release of 2-nitro-5-mercaptobenzoic acid (TNB) having a molar extinction coefficient of $\varepsilon_{412} = 13{,}600\ \mathrm{M^{-1}\,cm^{-1}}$, and corrected for the blank (buffer and DTNB only). Aliquots of the reaction mixture were removed at timed intervals after the addition of DTNB and assayed for residual activity. For the reactivation of DTNB-inactivated OSC, an appropriate aliquot of a 100 μM DTT solution in 5 mM phosphate buffer, pH 7.4, was added to DTNB-modified OSC solution and the reaction mixture was incubated at 37 °C for 2 h. After solvent extraction and TLC, OSC activity was calculated as previously described.

Inactivation of OSC by diethyl pyrocarbonate and reversal by hydroxylamine

Stock solutions of diethyl pyrocarbonate (DEPC) (10 mM) were prepared in absolute ethanol solution immediately before use. Chemical modification of OSC was initiated by the addition of various concentrations (0, 20, 40, 60, 80, and 100 μM final concentration) of an ethanol solution of DEPC to 0.5 mg/mL solutions of enzyme in 5 mM potassium phosphate, pH 7.0, and 0.5% Triton X-100. The reaction mixtures were incubated at 37 °C for various lengths of time and the excess reagent was then quenched by the addition of a 30-fold molar excess of L-histidine. A reaction mixture containing only enzyme, aliquots of ethanol equivalent to those used to add DEPC, and buffer was included as the uninhibited control for each modification experiment. At different times of modification, aliquots of reaction mixtures were removed and assayed for residual activity as previously described. The number of histidine residues modified by DEPC was calculated from the increase in absorbance at 240 nm using a molar extinction coefficients $\varepsilon_{240} = 3200\ \mathrm{M^{-1}\,cm^{-1}}$ for *N*-carbethoxyhistidine. In experiments using hydroxylamine to assess the reversibility of OSC modification by DEPC, purified OSC (0.5 mg/mL) was incubated at 37 °C with 0.08 mM DEPC and then diluted 1:1 with 1 M hydroxylamine, pH 7.0. After incubation at 37 °C for various lengths of time, activity was assayed as previously described. Control OSC activity was measured in parallel incubations without DEPC, but including the appropriate volume of hydroxylamine or phosphate buffer. Hydroxylamine treatment alone did not change OSC activity.

Results and discussion

Purification of bovine liver OSC

To date, no more than five oxidosqualene cyclases, mainly CAS and β-amyrin synthase from higher plants, have been purified to homogeneity. Few reports regarding the biochemical properties, partial protein structural information, and catalytic sites are currently available [9,23–26,34,35]. Motivation to elucidate the complex enzymatic cyclization/rearrangement mechanisms of oxidosqualene provoked us to purify the oxidosqualene-lanosterol cyclase from bovine liver and to characterize the putative amino acids involved in its catalytic activity. Using knowledge from the oxidosqualene-lanosterol cyclase, a membrane-bound protein found in both hog and rat livers, microsomes from bovine liver homogenates were treated with various concentrations of Triton X-100. The specific activity of the starting microsomal suspension was significantly higher than that obtained when the buffer contained 0.5% (w/v) Triton X-100. Therefore, the concentration of detergent was maintained at this level for subsequent purification steps to maintain enzymatic activity. In addition, a purification schedule was devised to obtain a higher yield and increased OSC activity over the crude solubilized microsomes. It was observed that after the solubilized enzymes are separated from the microsomes, they were stable for at least 2 weeks in Tris buffer (20 mM, pH 7.4) containing 0.5% Triton X-100, 1 mM DTT, 1 mM EDTA, 1 mM benzamidine, and 40 μg/mL PMSF at −80 °C without significant loss of enzyme activity.

Following recovery of OSC from the microsomal fraction, the crude enzyme solution was subjected to chromatographic separation using Q-Sepharose Fast Flow, hydroxyapatite, and HiTrap heparin columns. The enzyme adsorbed onto the Q-Sepharose Fast Flow column was eluted with IEB buffer containing 20, 50, and 100 mM sodium chloride, and the cyclase activity appeared in fractions containing 50 mM salt. Next, the fractionation of cyclase enzyme by hydroxyapatite chromatography showed that the cyclase enzyme was not retained on the column under the experimental conditions but the column effectively removed impurities. Finally, OSC was substantially purified by HiTrap heparin chromatography when eluted with HB buffer containing 50 mM potassium chloride. The hydroxyapatite and heparin steps significantly enhanced the fold of purification, yielding 728-fold enrichment in specific activity. Electrophoresis of the fractions after the dif-

Table 1
The purification of oxidosqualene-lanosterol cyclase from bovine liver

Fraction	Total protein (mg)	Total activity nmol/10 min	Yield (%)	Specific activity (pmol/min/mg)	Purification fold
Microsomal membranes	2417	348	100	2.4	1
Q-Sepharose FF	328	228	66	22	9
Hydroxyapatite	105	33.5	10	165	69
Heparin	9	16.6	5	1747	728

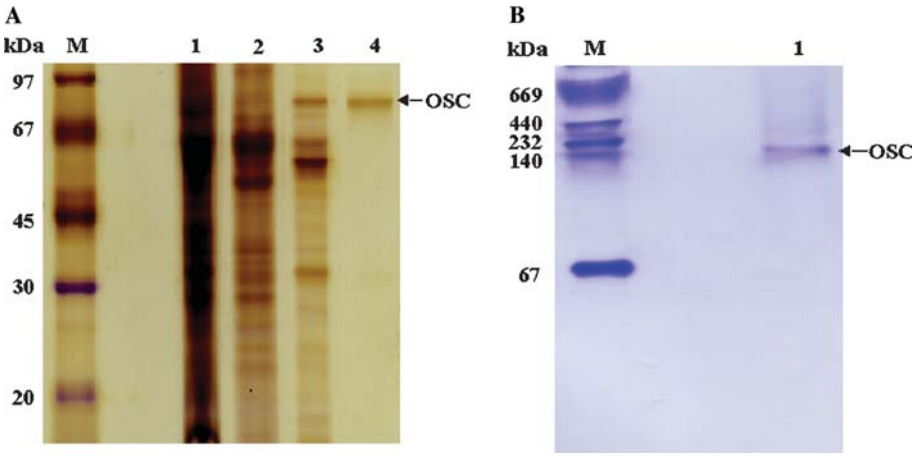

Fig. 2. (A) Silver-stained SDS–PAGE gel (10% cross-linked) of bovine liver OSC obtained from column chromatography fractions. Lane 1, marker proteins: phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa); lane 2, solubilized protein; lane 3, Q-Sepharose Fast Flow purified protein; lane 4, hydroxyapatite purified protein; and lane 5, HiTrap heparin purified protein. (B) Coomassie-stained non-denaturing PAGE gel of bovine liver OSC obtained from HiTrap heparin chromatography. Marker proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa).

ferent purification steps revealed a single protein band with a molecular weight of ~70 kDa, as visualized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by silver staining of the gels. A single band of about 140 kDa also was revealed when examined using non-denaturing polyacrylamide gel electrophoresis (PAGE). The purified homogeneous enzyme was stable for up to 6 months at −20 or −80 °C in the presence of Triton X-100, DTT, and phosphate buffer. As shown in Table 1 and illustrated in Fig. 2, the HiTrap heparin chromatography, which was applied to purify oxidosqualene cyclase for the first time, provides an effective method of purifying multi-milligram quantities of apparently homogeneous mammalian oxidosqualene cyclases.

Identification of OSC by tandem mass spectrometry

The purified protein then was subjected to *N*-terminal sequencing and internal amino acid determination to confirm that the isolated protein is indeed OSC. Multiple attempts to obtain the N-terminal sequence of OSC were unsuccessful, suggesting that the N-terminus of the protein may be blocked. For internal amino acid determination, the SDS–PAGE band corresponding to OSC was subjected to in-gel digestion using trypsin, as described by Rosenfeld et al. [36]. After digestion, the resulting peptide mixtures were analyzed by nanoscale capillary LC/MS/MS. A tandem mass spectrum of a doubly charged precursor *m/e* 683.45 is shown in Fig. 3. Following a database search of the mass of the peptide and its fragment ions, one unique hit matching the human OSC peptide sequence was identified. The sequence was determined to be ^{163}ILGVGPDDPDLVR^{175} of human OSC. Another peptide, whose sequence was determined to be ^{495}NPDGGFATYETK^{506} from LC/MS/MS analysis, also matched the OSC protein. An MS BLAST search for homologous proteins identified a third sequence, ^{248}LSAEEGPLVQSLR^{260}. The BLAST search identified all high-scoring pair (HSP), -regions of high local sequence similarity between individual peptides in the query, and a protein sequence from the database entry. In summary, the results showed that three peptide fragments from in-gel digestion of purified bovine liver OSC were homologous to OSCs from human (EC 5.4.99.7), rat (EC 5.4.99.7), and mouse (EC 5.4.99.8). These data provided evidence that the identity of the purified protein was bovine liver OSC. Individual HSP-aligned sequences of high similarity are shown in Fig. 4.

a	86.10	199.18	256.20	355.27	412.29	509.35	624.37	739.40	836.45	951.48	1064.56	1163.63	1319.73
b	114.09	227.18	284.20	383.27	440.29	537.34	652.37	767.39	864.45	979.47	1092.56	1191.63	1347.73
	Ile	Leu	Gly	Val	Gly	Pro	Asp	Asp	Pro	Asp	Leu	Val	Arg
y	1365.74	1252.65	1139.57	1082.55	983.48	926.46	829.41	714.38	599.35	502.30	387.27	274.19	175.12
z	1348.71	1235.62	1122.54	1065.52	966.45	909.43	812.38	697.35	582.32	485.27	370.24	257.16	158.09

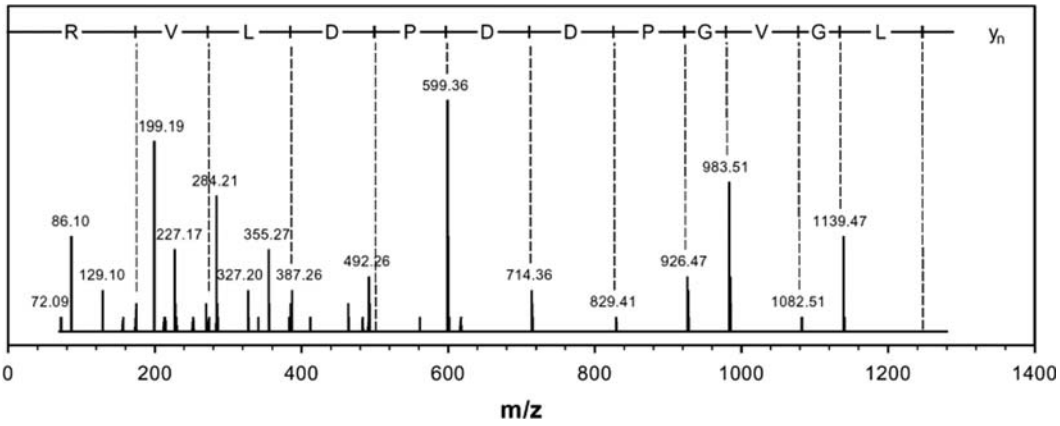

Fig. 3. Tandem mass spectrum of the doubly charged tryptic peptide at *m/z* 683.45 from purified bovine liver OSC protein. The complete amino acid sequence, ILGVGPDDPDLVR, was deduced from the mass differences in the y-fragment ion series and is also partly supported by b-ion and a-ion series. The sequence was shown using single letter amino acid symbols confirmed by a complete y-ion series in the MS/MS spectrum. Ions were labeled according to the nomenclature of Roepstorff and Fohlman. Underlined ions indicate fragment ions observed by the mass spectrometer.

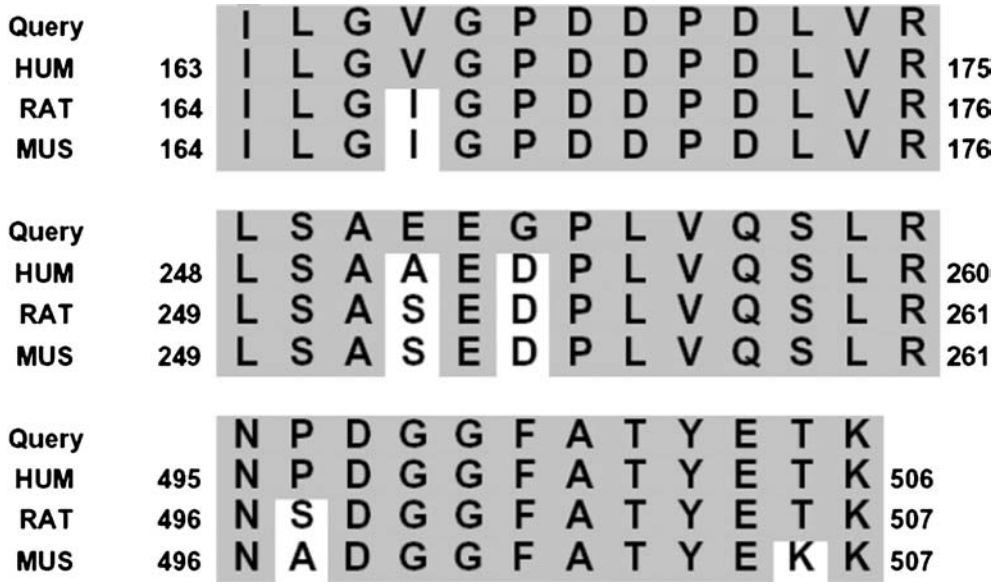

Fig. 4. Sequence alignment of trypsin-digested peptides from bovine liver OSC observed by tandem mass spectrometry. Three peptide sequences from purified protein of bovine liver detected by tandem mass spectrometry were homologous to lanosterol synthase (OSC) in human (EC 5.4.99.7), rat (EC 5.4.99.7), and mouse (EC 5.4.99.8). The consensus sequences are boxed in gray. The shaded amino acid residues indicate similarity between different species.

Biochemical characterization and inhibition studies of OSC

The biochemical properties of OSC were characterized further using the purified enzyme to investigate on kinetic parameters, temperature and pH optima, inhibition, and chemical modifications. Bovine liver OSC activity was tested at various pH values, using the following buffers (5 mM): citrate (pH 4.0, 5.0, and 6.0), Tris–HCl (pH 6.0, 7.0, 7.4, 8.0, and 9.0), potassium phosphate (pH 6.0, 7.0, 7.4, 8.0, and 9.0), and CAPS (pH 9.0 and 10.0). The results indicated that the purified enzyme possesses an optimal activity around pH 7.4 (Fig. 5). The temperature for optimal enzyme activity

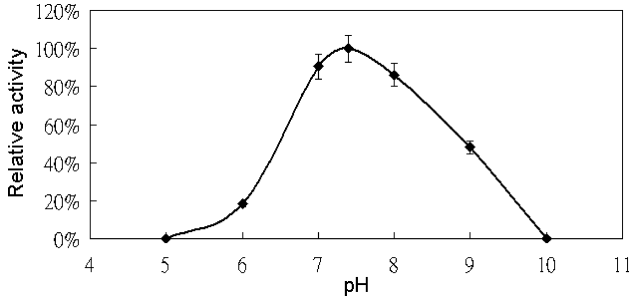

Fig. 5. pH dependence of bovine liver OSC activity. Fifty microliters (0.5 mg/mL) of purified OSC was added to the standard assay mixture (total volume 200 μL) containing 5 mM potassium phosphate (pH 7.4), 0.5% Triton X-100, 50 μM [*R*,*S*]2,3-oxidosqualene plus 5 mM citrate, Tris–HCl, potassium phosphate, or CAPS buffers so that the final pHs of the assay mixtures were 4.0, 5.0, and 6.0 (citrate); 6.0, 7.0, 7.4, 8.0, and 9.0 (Tris HCl); 6.0, 7.0, 7.4, 8.0, and 9.0 (potassium phosphate); 9.0 and 10.0 (CAPS); buffer ion effects were not present. The assays were performed as described under "Materials and methods" and expressed as percentage of control activity, which was determined under standard assay conditions.

was experimentally determined by incubation of OSC with [^{3}H](3*R*,*S*)-2,3-oxidosqualene in phosphate buffer (pH 7.4) at different temperatures (25, 37, 50, 60, and 70 °C) for 30 min intervals. Optimal activity was observed between 25 and 50 °C, with a maximum at 37 °C. The apparent K_M and k_{cat}/K_M values for the true substrate (3*S*)-2,3-oxidosqualene were determined to be 11 μM and 1.45 $mM^{-1}\,min^{-1}$, respectively. These data are consistent with those of cyclases obtained from other species [25,26,37–39]. However, the $K_{M(app)}$ for bovine liver OSC might be much larger than the true K_M, since a substantial portion of the substrate might end up in micelles but not enzyme bound [26]. Similarly, the relatively small value of k_{cat} may not reflect the real rate of the chemical reaction. Alternatively, these results may suggest a structural role for sterol in membrane function.

To shed light on inhibitory activity of the OSC-catalyzed cyclization/rearrangement reaction, a series of benzophenone-containing inhibitors, including Ro48-8071 from Hoffmann-La Roche, *N*,*N*-dimethyl-*N*-(4-methylenebenzophenonyl)amine, and a group of *N*-(*X*-methylenebenzophenonyl)pyridinium bromides (where $X = 2$, 3, and 4), were designed and synthesized as reported in the literature [6,22]. The benzophenone-containing OSC inhibitor, Ro48-8071, previously had been demonstrated to be one of the most potent inhibitors for a variety of liver OSCs, including human, rat, and monkey [6]. Specifically, the pyridinium and amine derivatives of benzophenone were designed to mimic the cationic monocyclic transition state and to provide a π-stacking interaction with electron-rich side chains of aromatic residues within OSC active sites. The abundance of aromatic residues within various cyclase enzymes led Griffin and Poralla to suggest an "*Aromatic Hypothesis*" that electron-rich aromatic or negative charge side chains might constitute the axial negative charges required to lower transition state barriers and to control the regio- and stereochemistry of the oxidosqualene cyclase-catalyzed cyclization/rearrangement reaction [7,20,21,40–43]. As expected, both Ro48-8071 and *N*-(4-methylenebenzophenonyl)pyridinium bromide are potent inhibitors of bovine liver OSC with IC_{50} values of 11 nM and 0.79 μM, respectively (Fig. 6). *N*,*N*-dimethyl-*N*-(4-methylenebenzophenonyl)amine, *N*-(3-methylenebenzophenonyl)pyridinium bromide, and *N*-(2-methylenebenzophenonyl)pyridinium bromide showed moderate inhibitory activity of 5, 16, and 59 μM, respectively, against the purified enzyme. The IC_{50} value of 11 nM for Ro48-8071 on bovine liver OSC suggested that it is a potent inhibitor of bovine liver OSC. Additionally, Ro48-8071 showed the most potent inhibitory activity with a trend of decreasing potency from the *para*-substituted pyridinium or amine to that of *meta*- and *ortho*-substituted regio-isomers of pyridinium derivatives. In agreement with the inhibition of both rat and bovine liver OSCs by Ro48-8071, potent inhibition of rat liver OSC also was observed for compounds containing lipophilic alkylammonium moieties of different chain lengths, thus making it a general phenomenon for cyclase inhibitors [44]. In addition, in the case of pyridinium derivatives, the *para*-substituted compound inhibits the bovine liver enzyme at a concentration approximately 75-fold less than the analogous *ortho*-substituted compound. This fact indicates that flat and stretched molecules such as the *para*-substituted

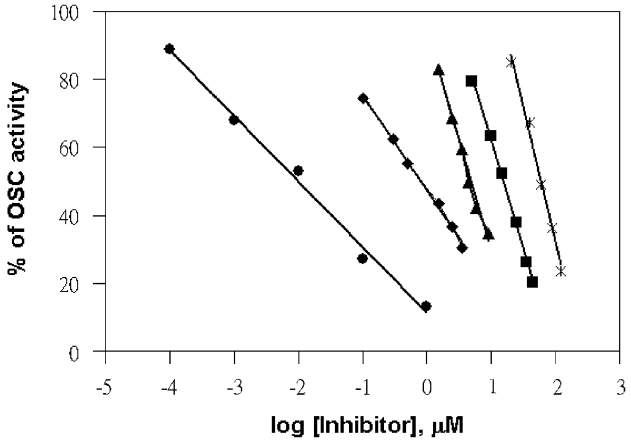

Fig. 6. The IC_{50} determination of bovine liver OSC by benzophenone-containing inhibitors. The HiTrap heparin purified OSC was pre-incubated at 37 °C in phosphate buffer (pH 7.4) in the presence of various concentrations of Ro48-8071 (●), *N-(4-methylenebenzophenonyl)pyridinium bromide* (◆), *N*,*N*-dimethyl-*N*-(4-methylenebenzophenonyl)amine (▲), *N*-(3-methylenebenzophenonyl)pyridinium bromide (■), and *N*-(2-methylenebenzophenonyl)pyridinium (*). The enzymatic activity was determined as the percentage of the activity obtained after pre-incubation in the absence of inhibitors. The percentage of OSC activity was plotted versus the logarithm of inhibitor concentrations.

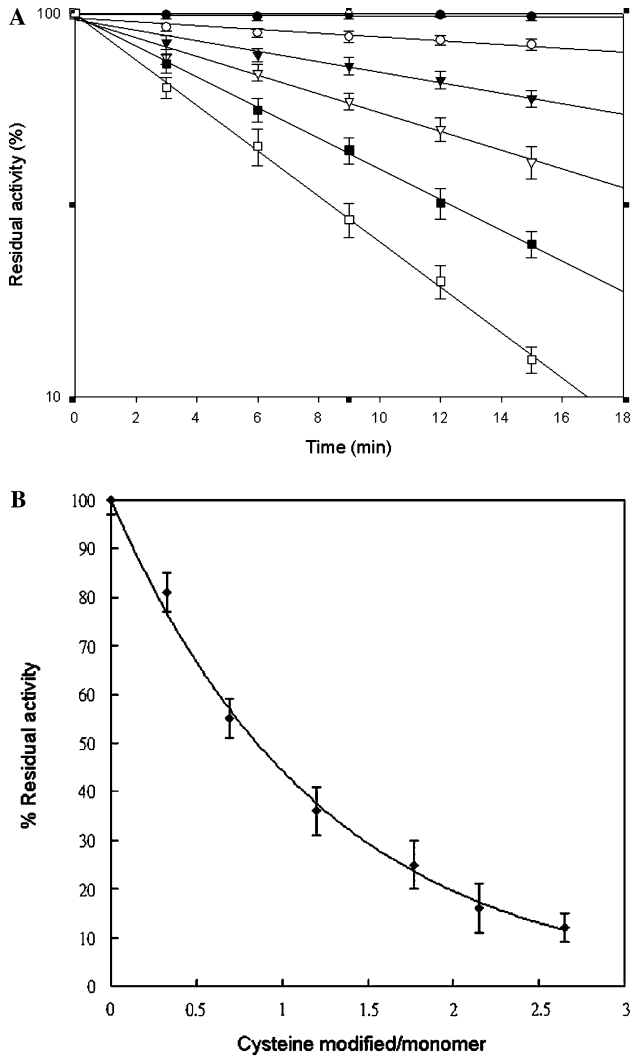

Fig. 7. Chemical modification of bovine liver OSC with DTNB. (A) Inactivation of OSC activity by DTNB. The purified OSC was incubated at 37 °C and pH 7.4 with various concentrations (0 μM (●), 20 μM (○), 40 μM (▼), 60 μM (▽), 80 μM (■), and 100 μM (□)) of DTNB reagent as described under "Materials and methods." Aliquots were removed at various time intervals for determining residual activity. After lipid extraction and TLC, the residual OSC enzyme activity was calculated as previously described. (B) Stoichiometry of DTNB-mediated inactivation. The purified OSC was treated with 50 μM DTNB at 37 °C and pH 7.4, as described under "Materials and methods." The number of modified cysteins was calculated from the differential absorbance at 412 nm in a parallel experiment under identical conditions. The error bars represent means ± SD of three independent determinations.

compound imitate the nature of the oxidosqualene during cyclization better than relatively bent molecules such as the *ortho*-substituted compound, which fit with less affinity into the enzyme cavity. Moreover, it seems reasonable to suggest that a common inhibition mechanism but different affinities of Ro48-8071, pyridinium, and amine-derived inhibitors for the OSCs can be attributed to the shape, size, or regio-chemistry of the inhibitors, but alternative explanations exist.

Chemical modifications

Chemical modification of amino acids yields meaningful information about the putative active-site structure, intramolecular conformational change, or mechanistic insights of OSC-catalyzed cyclization/rearrangement reactions. Previous work on chemical inactivation of both prokaryotic squalene cyclase and eukaryotic oxidosqualene cyclases suggested that a cysteine or histidine residue is essential for cyclase activity [12,45–50]. The cysteine-modifying agent, 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB), was used to assess the possible role of cysteine residues in the enzymatic function of bovine liver OSC activity [51,52]. As depicted in Fig. 7A, DTNB-inactivated bovine liver OSC in a time- and dose-dependent manner. Besides, correlation between the loss of enzymatic activity and the number of cysteins modified showed that ~90% inactivation was observed when 2.6 ± 0.5 mol of DTNB reacted with 1 mol of OSC, as shown in Fig. 7B. Two essential cysteine residues responsible for catalysis could be estimated from kinetics of inactivation experiments. To assess whether modification is active-site-directed, cysteine modification accompanied by substrate protection on OSC activity was carried out. The purified OSC was incubated with DTNB both in the presence and absence of substrate and the effect of protection on inactivation was examined. No apparent difference in the release of TNB was observed when purified OSC was reacted with DTNB both in the presence and absence of substrate. This result indicated that the sulfhydryl group essential for catalysis is probably not located in the substrate entrance channel or binding pocket but somehow alternates enzyme's conformation, making it less susceptible to substrate binding, but alternative explanations exist. Reactivation of DTNB-inactivated OSC was determined by adding 100 μM of DTT to various concentrations of DTNB-inhibited OSC and measuring the amount of lanosterol produced. The TLC results showed ~30% more lanosterol was produced in the presence of DTT than in the absence of DTT, indicating that inactivation was due to modification of cysteine(s) and OSC could be cycled reversibly through inactive and active states by oxidation (DTNB) and reduction (DTT) of cysteine residues. In parallel, to gain insight into the structural correlation between Ro48-8071 and DTNB binding sites, purified OSC was again incubated with DTNB both in the presence and absence of the inhibitor. A substantial decrease both in absorbance at 412 nm and lanosterol formation was observed, indicating that both Ro48-8071 and DTNB may compete for a similar binding pocket, conformational alterations of the enzyme, or that the higher binding affinity for Ro48-8071 may impede the binding of DTNB to OSC. However, precise determination of the locations of cysteine residues

involved in the catalysis awaits ultimate OSC structure elucidation.

Considering the likely sequence similarity between (oxido)squalene cyclase genes from different species, it will be of considerable interest to determine whether functional amino acid residues at their active sites have been conserved during molecular evolution. Clearly, histidine is a reasonable candidate to serve as a general acid or general base, both of which enhanced activity of the active-site aspartate residue in the activation of the double bond or oxirane ring for cyclization initiation [17]. In addition, histidine stabilized carbocation formed during the OSC-catalyzed cyclization/rearrangement process [12]. Although the purification and characterization of OSC from bakers yeast failed to identify any histidine residues involved in catalytic function, site-directed mutagenesis of *Saccharomyces cerevisiae* OSC revealed at least two histidine residues that are essential for catalytic function [12,48]. Recent site-directed analyses of *S. cerevisiae* OSC and *Arabidopsis thaliana* CAS from our laboratory also support the participation of at least one histidine residue in the OSC-catalyzed cyclization/rearrangement reaction (unpublished results). To explore further the possible involvement of histidine residues in bovine liver OSC catalytic function, we performed the site-specific modification of this residue with various concentrations of the histidine-specific agent diethyl pyrocarbonate (DEPC) and reversed the histidyl-DEPC reaction with hydroxylamine. As depicted in Fig. 8A, treatment of purified bovine liver OSC with DEPC resulted in a time- and concentration-dependent inhibition of OSC activity, which is consistent with inactivation of the squalene-hopene cyclase observed with this reagent [46]. The stoichiometry of inactivation of OSC by DEPC was measured from the absorption change at 240 nm using a molar extinction coefficient of 3200 M^{-1} cm^{-1} [53]. As shown in Fig. 8B, plots of residual enzyme activity as a function of the number of histidine residues modified by DEPC at pH 7.0 showed that modification of about two histidine residues per subunit resulted in complete inactivation of the enzyme. Additionally, to further distinguish histidine from tyrosine or primary amino functional group such as lysine as the active-site residue modified by DEPC, purified OSC was treated at pH 6–9. The result showed that maximal OSC inhibition was observed near neutral pH. The histidine then was regenerated by treatment with neutral hydroxylamine (1 M, pH 7.0) and followed by an OSC assay. As shown in Fig. 9, DEPC modification of bovine liver OSC at pH 7.0 followed by OSC assay resulted in OSC inhibition and the enzymatic activity of the inhibited OSC was restored to the extent of 43% of the unmodified control enzyme upon the addition of hydroxylamine. These data collectively support the fact that DEPC inactivates OSC by the modification of histidine residue(s), which is critical for the catalysis. Moreover, the partial but not full reactivation of DEPC-

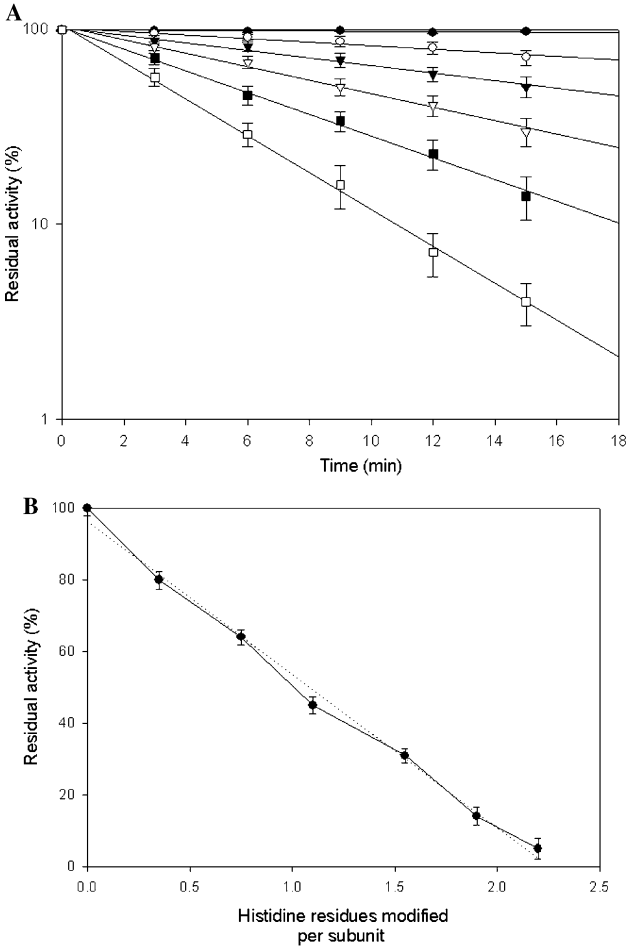

Fig. 8. Chemical modification of bovine liver OSC with DEPC. (A) Time-dependent inactivation of OSC by DEPC. The purified OSC (2 μM) was incubated with various concentrations (0 μM (●), 0.02 mM (○), 0.04 mM (▼), 0.06 mM (▽), 0.08 mM (■), and 0.1 mM (□) of DEPC at 37 °C. At various time intervals, aliquots were withdrawn, mixed with histidine solution, and assayed for the residual activity as described under "Materials and methods." (B) Purified OSC (0.5 mg/mL) was incubated at 37 °C and pH 7.0 with 2 mM DEPC, and aliquots were removed at intervals for assessment of absorbance of the enzyme at 240 nm and for assay of residual enzyme activity. The error bars represent means ± SD of three independent determinations.

inactivated OSC activity by hydroxylamine indicated either di-substitution of the imidazole by DEPC or instability of bond linkage between carbethoxyl group and histidine residues, with subsequent ring cleavage during reversal with hydroxylamine. On the other hand, the presence of other nucleophiles such as tyrosine, cysteine, and primary amino group of lysine, which might contain an abnormally low pK_a for the aforementioned function, has not been entirely excluded [53]. Therefore, the precise residue and its functional role identified in this study await further elucidation by cloning/sequencing of the corresponding gene and site-directed mutagenesis. These studies currently are under investigation.

In summary, the present results lead to several valuable conclusions about bovine liver OSC. First, a highly

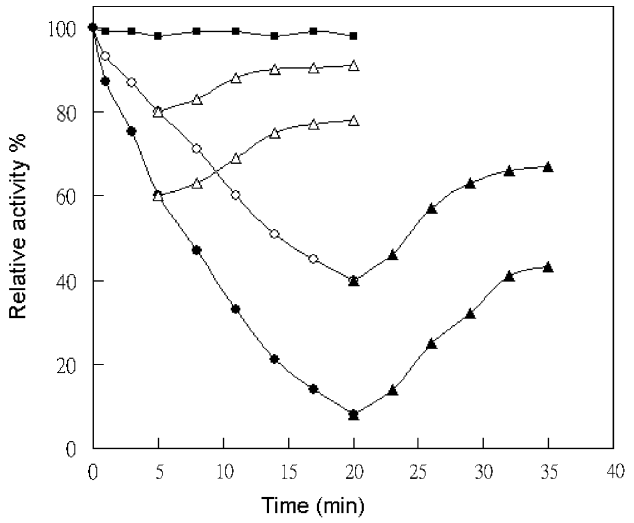

Fig. 9. Restoration of enzymatic activity of DEPC-inactivated OSC by hydroxylamine. Purified OSC (0.5 mg/mL, pH 7.0) was incubated with 0 (■), 0.04 (○), and 0.08 (●) mM DEPC at pH 7.0 and 37 °C. After 5 (△) and 20 (▲) min, 1 M hydroxylamine was added to the mixture. Samples were taken at different times for the determination of recovery of enzymatic activity.

stable and homogeneous OSC protein can be prepared reproducibly from solubilized bovine liver microsomes. Second, biochemical and biophysical determinations of bovine liver OSC thus confirm its identity. Third, several benzophenone-containing inhibitors exhibited potent to moderate inhibitory activity against the purified protein. Fourth, chemical modifications of bovine liver OSC confirmed the importance of cysteine and histidine residues in OSC catalytic function or conformation. Finally, these results should lead to the initiation of work on cloning and sequencing of the corresponding gene, screening of OSC inhibitors using this enzyme as a potential target, and structural determination for detailed mechanistic elucidation of the OSC-catalyzed cyclization/rearrangement reaction.

Acknowledgments

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