J. Biochem. Mol. Biol. Vol. 30, No. 5, pp. 370~377 September 30, 1997

Cholesterol Biosynthesis from Lanosterol: Development of a Novel Assay Method, Characterization, and Solubilization of Rat Hepatic Microsomal Sterol Δ^7-Reductase

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(Received August 6, 1997)

Abstract : A novel assay method is described for rapid quantitation of reaction rate of sterol Δ^7-reductase (Δ^7-SR) which catalyzes reduction of the Δ^7-double bond of sterols. Of six different organ tissues-liver, small intestine, brain, lung, kidney, and testis-, Δ^7-SR activity was detected only in liver (2.30 nmol/min/mg protein) and testis (0.11 nmol/min/mg protein). Using a newly developed method which employs diet-induced enzyme proteins and ergosterol as substrate, we assessed both kinetics (K_m=210 μM, V_{max}=1.93 nmol/min/mg) and inhibition of the rat hepatic Δ^7-SR against well-studied cholesterol lowering agents such as triparanol (IC_{50}=16 μM), 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) (IC_{50}=5.2 μM), and trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944) (IC_{50}=0.25 μM). Of the three well-known AY-9944-sensitive cholesterogenic enzymes (i.e., Δ^7-SR, sterol Δ^8-isomerase, and sterol Δ^{14}-reductase), Δ^7-SR was found to be the most sensitive enzyme with a noncompetitive inhibition of this compound (K_i=0.109 μM). Substrate specificity studies of the microsomal Δ^7-SR indicate that the relative reaction rate for 7-dehydrocholesterol and ergosterol are 5.6-fold and 1.6-fold higher than that for lathosterol. Δ^7-SR activity was also modulated by feeding rats a diet supplemented with 0.5% ergosterol (>2.6-fold) in addition to 5.0% cholestyramine plus 0.1% lovastatin (≃5.0-fold). Finally, microsomal Δ^7-SR was solubilized by 1.5% 3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and enriched on PEG (0~10%) precipitation, which should be suitable for further purification of the enzyme.

Key words : cholesterol biosynthesis, 7-dehydrocholesterol, ergosterol, sterol Δ^7-reductase

As the final step in cholesterol biosynthesis from lanosterol, the reduction of the Δ^7-double bond of sterol intermediates is catalyzed by sterol Δ^7-reductase (Δ^7-SR) in the presence of reduced pyridine nucleotide under anaerobic conditions (Kandutch, 1962; Dempsey *et al.*, 1964; Dempsey, 1965; Dempsey, 1969; Gaylor, 1981; Trzaskos and Gaylor, 1985). Stereospecificity studies have shown that sterol Δ^7-reduction involves a *trans*-addition of 7α- and 8β-hydrogens which are derived from NADPH and water, respectively (Wilton *et al.*, 1968). Some studies on the properties of Δ^7-SR (Ritter and Dempsey, 1970) and molecular cloning of cDNA encoding plant Δ^7-SR (*Arabidopsis thaliana*) (Lecain *et al.*, 1996) have been reported.

It is well known that Δ^7-SR activity is strongly inhibited by trans-1,4-bis(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944) (Dvornik and Hill, 1968) which also inhibits sterol Δ^{14}-reductase (Δ^{14}-SR) (Paik *et al.*, 1984; Kim *et al.*, 1995) and sterol Δ^8-isomerase (Δ^8-SI) (Paik *et al.*, 1986; Kang *et al.*, 1995), termed AY-9944 sensitive cholesterogenic enzymes (ASCE). Studies on inhibition of plant Δ^7-SR (maize) activity by 6-aza-B-homosteroids and their analogues have been reported (Rahier *et al.*, 1996). However, further detailed investigations on the mammalian Δ^7-SR with respect to mechanism of regulation, diet-mediated induction and substrate specificity have not been reported.

A deficiency of Δ^7-SR activity in humans has been known to cause Smith-Lemli-Opitz syndrome (SLOS), which is an autosomal recessive disorder, and is characterized by elevated 7-dehydrocholesterol in serum (Smith *et al.*, 1964; Irons *et al.*, 1994; Tint *et al.*, 1994; Tint *et al.*, 1995; Alley *et al.*, 1995; Honda *et al.*, 1995; Wolf *et al.*, 1996). Although a diagnositc system using gas liquid chronatography-mass spectrophotometry (GLC-MS) techniques for determining the 7-dehydrocholoesterol level in plasma, amniotic fluid and cultured skin fibroblasts has been reported (Kelly, 1995), a direct analysis of its enzymic reaction in tissues has been hampered probably due to a lack of a convenient and rapid

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assay method that uses a commercially available unlabeled sterol intermediate (e.g., ergosterol). The currently available method is mainly based on a cumbersome assay procedure using radioisotope-labeled 7-dehydrocholesterol as a substrate.

The purpose of this work is: 1) to develop a novel assay method using commercially available ergosterol as substrate; 2) to investigate the mechanism of enzyme regulation *in vitro* as well as *in vivo*; and 3) to solubilize the membrane-bound Δ^7-SR from the microsomes. Our data showed that rat hepatic Δ^7-SR is regulated by the presence of several cholesterol-lowering agents (*in vitro*) and by feeding one of its substrates (ergosterol) (*in vivo*), and can be solubilized in active form by detergent, making it suitable for further purification of the enzyme.

Materials and Methods

Animals and diet feeding

Male Sprague-Dawley rats (200~250 g body weight) were maintained on a standard rodent chow under a reverse light cycle (light 6:00 p.m-6:00 a.m.; dark 6:00 a.m. ~6:00 p.m.) as previously described (Kim *et al.*, 1995). In the diet feeding experiments, each diet-fed group (3 to 4 rats per group) had been fasted for 12 h and then fed diets supplemented with various agents such as 5% cholestyramine plus 0.1% lovastatin (CL-diet), and ergosterol (e.g., 0.1%, 0.5%, and 1.0%).

Reagents, drugs, and other materials

The sources of the following drugs or agents are indicated in parentheses. AY-9944 (Dr. D. Dvornik at Wyth-Ayerst, Princeton, NJ), cholestyramine (LG Chem, Pharmaceutical Division, Seoul, Korea), Squalestatin I (Dr. B. M. Bain at Glaxo Research & Development, Middlesex, U.K.), Triparanol (Dr. H. W. Bohme at Marion Merrel Dow Research Institute, Cincinnati, OH), 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A) (Dr. R. J. Cenedella at Kirksville College of Osteopathic Medicine, Kirksville, MO), and Lovastatin^R (Dr. Y-K. Sim at Choongwae Phamaceutical Co., Suwon, Korea). Ketoconazole was obtained from Korea Janssen Pharmaceutical Co. (Seoul, Korea). $NADP^+$ and NADPH were obtained from Sigma. Sterols including ergosterol, 7-dehydrocholesterol, lathosterol and cholesterol were from Steraloid. Collagenase was obtained from GIBCO, and most of the other tissue culture media and supplements were from Sigma. All other reagents were the best grade available.

Procedures of microsome preparation and standard enzyme assays

Rats were killed by decapitation at the midpoint of the dark period (10 p.m.) and their livers were excised and processed for microsome preparation as previously described (Kim *et al.*, 1995). A standard assay for Δ^7-SR was carried out as follows. Three hundred seventy nanomoles of substrate (ergosterol, 7-dehydrocholesterol, or lathosterol, 5α-cholestra-7-en 3β) in suspension that had been made by the addition of Tween-80 (70:1= detergent:sterol) was added to an assay mixture (total volume 1.0 ml of 0.1 M Tris-HCl buffer [pH 7.4] containing 1 mM glutathione [reduced form], 0.5 mM EDTA, 20% glycerol w/v, 2 mg protein, 2 mM NADPH, 25 mg glucose and 20 units glucose oxidase, designated as 'Buffer A') that had been preincubated under nitrogen at 37°C for 4 min. The complete mixture was then incubated anaerobically in sealed flasks for 10 min at 37°C unless otherwise indicated. Incubation was terminated by the addition of 1 ml of methanolic KOH followed by heating under reflux for 10 min. Sterols were extracted with 3 volumes of petroleum ether and dried under N_2 gas followed by GLC analysis (see below). Enzymic activity was calculated from the relative amounts of substrate and product in incubated samples compared with unincubated controls. Throughout this study the only chromatographically detectable endogenous sterol of consequence in microsomal protein was cholesterol. For the *in vitro* inhibition experiments, drugs or agents were dissolved in dimethyl sulfoxide (DMSO) such that the final concentration of DMSO was less than 0.3% (w/v) of the incubation mixture.

Preparation of rat hepatocytes and enzyme assay

Spraque-Dawley rats (male, 250~300 g body weight) were fed the normal diet or the CL-diets for 7 days. Liver perfusion was performed as described (Kim *et al.*, 1995). Hepatocytes (5×10^6 cells/plate) were plated on 100 nm collagen-coated (2.5 μl/cm^2 of 2 mg/ml collagen) tissue culture dishes which contained 10 ml of Wymouth MB 752/1 media. Cells were incubated at 37°C under an atmosphere of 95% air (v/v) and 5% CO_2 (v/v) in culture medium, and the medium was changed 3 to 4 h after plating. Cell viability was determined by the trypan blue exclusion assay. After 20 h incubation, cells were harvested for the measurement of enzyme activity. Enzyme assay was performed using the total cell extracts (0.5 to 2 mg) as described above. Protein concentration was determined by the Lowry's method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Solubilization of the membrane-bound enzyme

All procedures were conducted at 4°C unless otherwise stated, as previously described (Paik *et al.*, 1986). Washed microsomes were suspended in a protein concentration of 20 mg/ml in Buffer A. 4% (w/v) solutions of 3-

[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were made fresh with cold Buffer A, and detergent solution was added dropwise to the protein suspension such that the final detergent ratio, with respect to protein, was 1.5:1. Additional buffer solution was added to adjust the final detergent concentration to approximately 1.5% (w/v) before centrifugation. A clear supernatant fraction (S-1) was obtained by centrifugation at 105.000×g for 1 h. This fraction was examined for Δ^7-SR activity.

Polyethylene glycol 3000 precipitation of the solubilized enzyme

A fresh 40% (w/v) solution of polyethylene glycol (PEG) 3000 (M_r=3000~3700) was prepared in Buffer A. An approximate volume of cold PEG solution was added in drops to the S-1 fraction and mixed well by gentle stirring on an ice bath. After the final addition, the protein suspension was stirred on ice for 2 h and then centrifuged to sediment the precipitated protein (35.000×g for 15 min). In this study, PEG precipitates were isolated from three fractions: P-A, 0~10%; P-B, 10~20%; P-C, 20~30%. The P-A fraction (0~10%) was used for further enrichment of Δ^7-SR activity. Enzymic activity was measured on a small aliquot of each PEG fraction which was suspened in fresh Buffer A.

Analytical procedures

GLC analysis was performed with a Hewlett-Packard 5890II GC using a capillary column (SAC-5, 5% diphenyl/95% dimethylsiloxane, 30 m×0.25 mm, 0.25 μm internal diameter) as described (Kim *et al.*, 1995). Sterols were quantitated by using a flame ionization detector. The relative retention times of sterols that were well separated on GLC (compared with a cholesterol standard) were: ergosterol, 1.162; 7-dihydroergosterol, 1.086; lathosterol, 1.098; 7-lathostanol, 1.033. GLC-MS analysis was conducted on a Hewlett-Packard 5988 GLC-MS system under conditions essentially as described previously (Shafiee *et al.*, 1986).

Results

Development of a novel assay method for Δ^7-reductase (Δ^7-SR) using ergosterol as substrate

Although radioactive 7-dehydrocholesterol has long been used as substrate for Δ^7-SR, we have attempted to use ergosterol for the enzyme assay because of its superior resolution on GLC analysis from 7-dihydroergosterol, the enzyme reaction product, and endogeneous cholesterol, which otherwise is a reaction product when 7-dehydrocholesterol is used as substrate. Fig. 1A shows clearcut separation of each sterol peak in GLC analysis

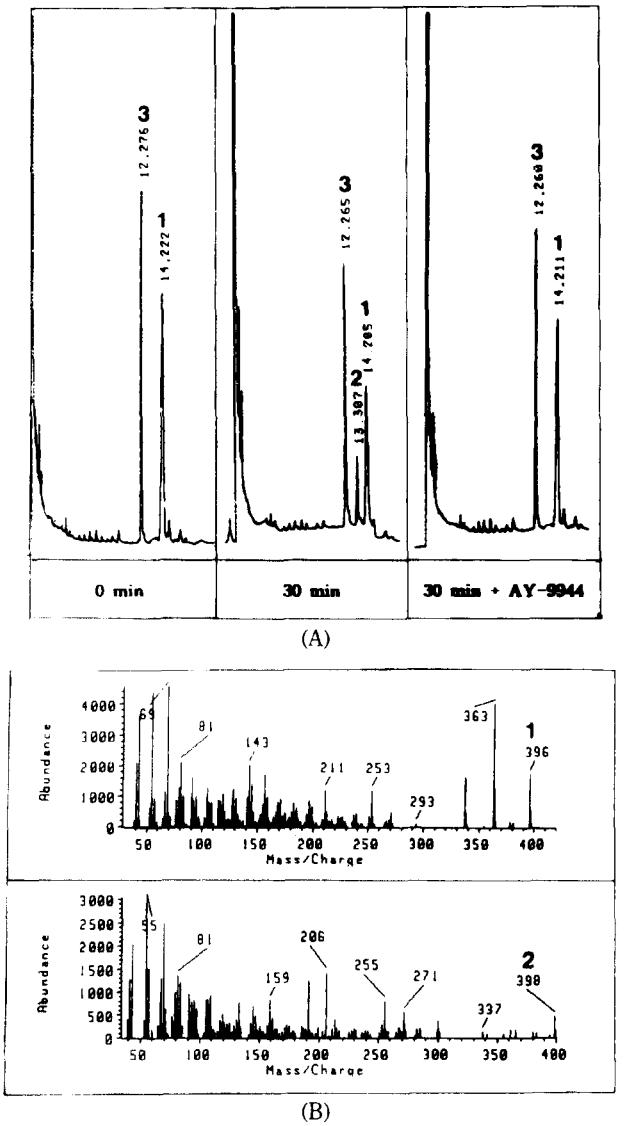

Fig. 1. Analysis of reaction products formed by sterol Δ^7-reductase. Assays were conducted anaerobically at 37°C using 370 μM ergosterol as substrate and 1.0 mg of microsomes that had been prepared from the CL-diet. A, GLC analysis of incubation products of 0 min (left), 30 min (middle), and 30 min in the presence of 10 μM AY-9944. Retention times of each sterol peak are indicated. B, GLC-MS analysis of reaction products obtained from 30-min incubation. Numbers 1 (panel A and Top of panel B) 2 (A and bottom of panel B), and 3 (panel A) indicate ergosterol (molecular ion=396), 7-dihydroergosterol (molecular ion=398) and cholesterol, respectively.

that was produced from a typical Δ^7-SR reaction when ergosterol was used a substrate in the presence of CL-diet induced microsomes. The product peak 2 (7-dihydroergosterol, or ergosta-5,22-dienol) is not present in the sample that had been treated by AY-9944, a potent inhibitor for the enzyme (Dvornik and Hill, 1968). GLC-MS analysis presented in Fig. 1B confirms that peaks 1 and 2 correspond to authentic ergosterol (mol-

ecular ion=396), and 7-dihydroergosterol (molecular ion =398), which are the substrate and the product of Δ^7-SR, respectively. This result reveals that ergosterol can be converted very efficiently to dihydroergosterol by rat liver microsomal Δ^7-SR.

In order to detect the enzyme activity easily, it was necessary to enhance enzymic activity by modulating diet. Therefore, we examined a diet-mediated induction of Δ^7-SR activity as seen in the case of other members of ASCE (e.g., sterol Δ^{14}-SR [Kim *et al.*, 1995], and 8-SI [Kang *et al.*, 1996]). Microsomes from rats that had been fed either the normal diet or the CL-diet were incubated with ergosterol as substrate. Approximately 6-fold induction of the enzyme activity was observed in microsomes (i.e., specific activity (S.A.)=0.38 [for the normal diet] vs 2.23 [for the CL-diet] nmol/min/mg protein at 10-min incubation point) (Fig. 2). An identical result was also obtained from freshly isolated hepatocytes that had been prepared from rats fed either the normal diet or the CL-diet (data not shown). Having established optimal assay condition, tissue distribution of the Δ^7-SR was examined using different tissue microsomes. Of six different organ tissues-liver, small intestine, brain, lung, kidney, and testis-enzyme activity was detected only in liver (2.30 nmol/min/mg protein) and testis (0.11 nmol/min/mg protein). Therefore, we used only liver microsomes for the entire study.

In the studies of enzyme properties using this method, the reduction of Δ^7-double bond of ergosterol catalyzed by Δ^7-SR has been shown to require NADPH (not NADH) as a proton donor (Lee and Paik, unpublished data). Although the reaction occurs under oxygen (average specific activity [S.A.]=1.30 nmol/min/mg protein), anaerobic conditions yield maximal rates of conversion (S.A.=2.23 nmol/min/mg protein). The enzymic activity is sensitive to sulfhydryl-binding agents such as N-ethylmaleimide (>98% inhibition at 1 mM), and not affected by the presence of metal ions (e.g., Mg^{2+}, Ca^{2+}, and Mn^{2+} etc.) (data not shown). Inactivation of the enzyme by N-ethylmaleimide was not recovered by the addition of glutathione (unpublished data).

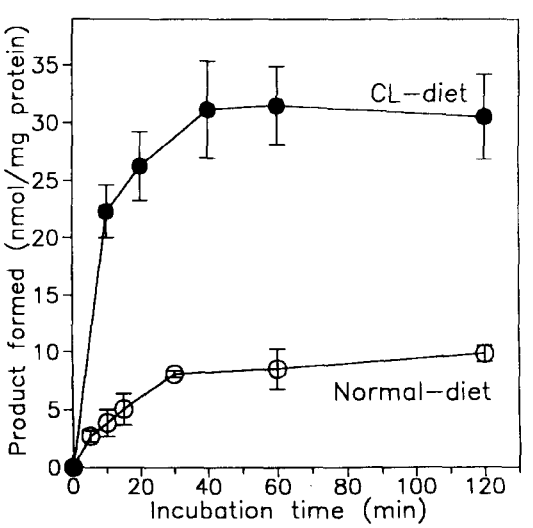

Fig. 2. Induction of sterol Δ^7-reductase activity by feeding cholesterol-lowering agents. Length of incubation was varied with 1.0 mg of microsomal protein. Values represent mean±S.D. of triplicate samples. Protein sources are: CL-diet (●), normal diet (○). Microsomes were prepared from a pool of 3 to 5 rats of each diet group.

Kinetic behavior and substrate specificities of Δ^7-SR

Fig. 3 shows progressive saturation of the enzyme with increasing ergosterol concentration. The apparent K_m and V_{max} values for the Δ^7-SR of ergosterol were calculated to be 210 μM and 1.93 nmol/min/mg, respectively. To investigate the substrate specificity of the enzyme, we compared the relative reaction rates of three selected sterols. We selected three representative sterol intermediates containing the 7(8)-double bond and examined the substrate preference for this enzyme. Fig. 4 shows a representative time course for the conversion of ergosterol, 7-dehydrocholesterol and lathosterol to their corresponding reduced sterols. Of the Δ^7-sterols examined for their specific activities at 5 min incubation point, 7-dehydrocholesterol (S.A.=9.13 nmol/min/mg) appears to be the most preferred substrate for Δ^7-SR, followed by ergosterol (S.A.=2.63 nmol/min/mg) and lathosterol (S.A.=1.66 nmol/min/mg).

Effects of cholesterol-lowering agents on Δ^7-SR activity

Although this enzyme has long been known to be inhibited by the well-known cholesterol-lowering agent,

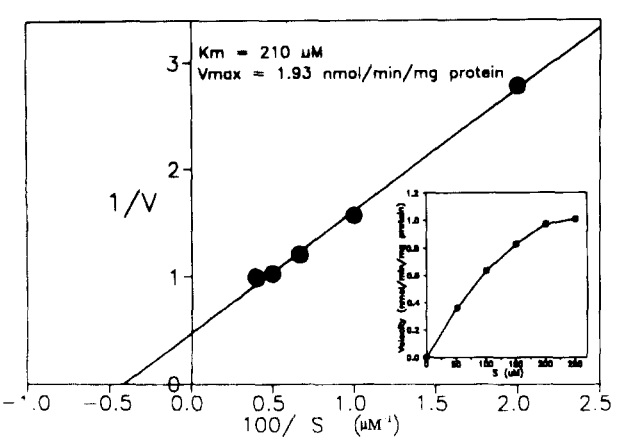

Fig. 3. Lineweaver-Burk reciprocal plot of microsomal sterol Δ^7-reductase. Assays were conducted anaerobically for 10 min using 1.0 mg protein (the CL-diet) under standard conditions with a varying amount of ergosterol.

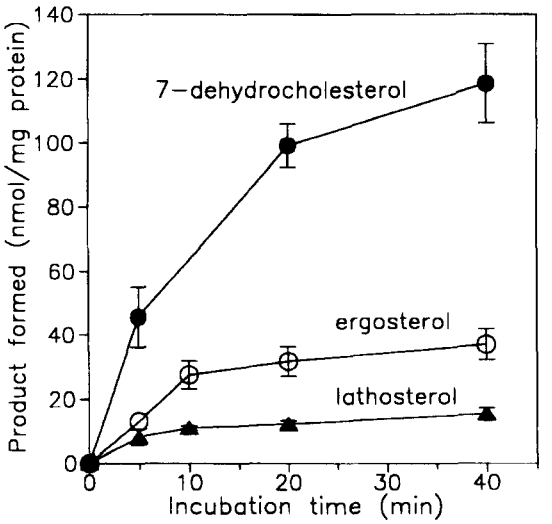

Fig. 4. Relative reaction rate of sterol substrates for Δ^7-reductase activity. Incubation length was varied with 1.0 mg of the CL-diet microsomal protein. Assays were conducted anaerobically at 37°C with 370 μM of each sterol substrate under the standard condition. Internal standard (5α-cholestane) was added into an incubation mixture for calibration of enzyme reaction products when 7-dehydrocholesterol was used as substrate. Values are mean (±S.D.) of triplicate samples. Sterol substrates are 7-dehydrocholesterol (●), ergosterol (○), and lathosterol (▲), respectively.

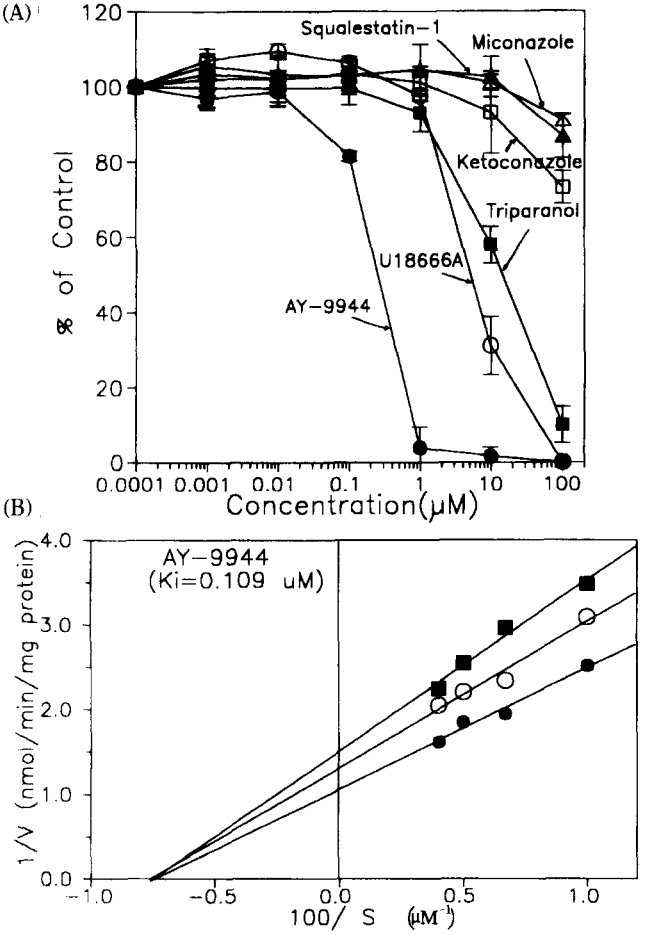

Fig. 5. Titration of Δ^7-reductase by various cholesterol-lowering agents *in vitro*. (A) Determination of IC_{50}s. Assays were conducted anaerobically at 37°C with 380 μM ergosterol in the presence of each agent such as. miconazole (△), squalestatin-1 (▲), triparanol (■), U18666A (○), and AY-9944 (●), respectively. Each agent, dissolved in DMSO (total 0.3% w/v), was added to the incubation mixture followed by addition of the substrate and continued incubation for 15 min. Enzyme assay of the control group was performed in the presence of an equal amount of vehicle solvent (DMSO). The relative inhibition by each drug was calculated based on the percent in reduction of the enzymic activity of the microsomes prepared from the rat fed CL-diet. Each point is the mean±(S.D.) of triplicate determinations. (B) Lineweaver-Burk reciprocal plot of Δ^7-reductase in the presence of varing amount of AY-9944. The concentration of AY-9944 was: 0 μM (●), 0.02 μM (○), 0.05 μM (■).

AY-9944 (Dvornik and Hill, 1968), its inhibitory concentrations (e.g., IC_{50}) as well as their mechanism of action against Δ^7-SR have not been reported. To examine how various cholesterol-lowering agents affect Δ^7-SR activity *in vitro*, and to compare their relative inhibitory potencies (if any) microsomes obtained from the rats fed the CL-diet were incubated in the presence of these agents. Fig. 5A shows the titration curve for some of these agents. The concentration of these agents required for 50% inhibition (IC_{50}) are estimated from the titration curve and marked as indicated. The relative IC_{50} values of AY-9944, U18666A and triparanol were estimated to be approximately 0.25 μM, 5.2 μM and 16 μM, respectively. Fig. 5B illustrates an estimation of the K_i value of AY-9944 (0.109 μM), the best known potent inhibitor for rat hepatic microsomal Δ^7-SR. As shown, the inhibition mode of Δ^7-SR by AY-9944 was determined to be noncompetitive. Analysis of the inhibition constants (K_i) of AY-9944 indicates that it has more than 1900-fold higher affinity for the enzyme as compared to that of ergosterol.

Solubilization and enrichment of sterol Δ^7-reductase

As an attempt to isolate an active Δ^7-SR from the membrane, microsomes were treated with various detergents and their combinations (Table 1). Enzyme activity was examined directly using S-1 resuspended in fresh Buffer A. An active form of soluble Δ^7-SR was detected in S-1 obtained using 1.5% CHAPS with near 90% recovery of activity as well as protein amounts (Table I). The NADPH-dependence of the enzyme both in S-1 and the microsome was essentially the same (data not shown). In both fractions, a maximum enzyme activity was observed at 2 mM NADPH from the titration curve. Both microsomal and solubilized Δ^7-SR

Table 1. A representative example of solubilization of the sterol Δ^7-reductase using various combinations of detergents

detergent	specific activity (nmol/min/ mg protein)	relative value[a] (%) (n=3)	protein recovery (%)
None (crude microsome)	1.216±0.032	100	100
CHAPS 0.5 %	0.850±0.020	69.42±0.975	68.4
1.0 %	1.076±0.042	89.29±1.800	80.6
1.5 %	1.078±0.020	89.40±1.135	93.1
2.0 %	1.038±0.051	86.14±4.023	86.4
OG+STDC	0	0	>95
OG+CHAPS	0	0	>95
NP-40 (0.5%)	0	0	>95
NP-40 (1.0%)	0	0	>95

[a]% relative values of enzyme activity (mean±S.D.) in S-1 fraction are those compared to the appropriate microsomal control incubated at the same time.

appeared to have a relatively broad pH profile in acidic region, whereas drastically decreased activity was observed with only moderate elevation of pH above 7.4 (data not shown). However, there is a slight difference in optimal pH values between S-1 (pH=6.4) and the microsomal enzyme (pH=7.4). Although the cause of this pH shift toward the acidic region is not known at present, it seems to be due to the presence of a zwitter ionic detergent such as CHAPS, which may act as a neutralizer for basic amino acid residues near the active site of the enzyme. This S-1 was stable for a week without significant loss of activity at 4°C. Enrichment of Δ^7-SR was attempted by precipitation of the S-1 using different concentrations of PEG3000. For the assay of the PEG fraction, each precipitated protein was resuspended in fresh buffer A. An active form of Δ^7-SR was obtained from 0~10% (P-A) fraction of PEG precipitation with a specific activity of 1.36 nmol/min/mg protein. Further purification and isolation of this PEG enriched fraction is now underway.

Regulation of Δ^7-SR activity *in vivo* by feeding ergosterol

It was of interest to examine whether the activity of Δ^7-SR could also be modulated *in vivo* by its substrate (ergosterol). Rats were fed with diet supplemented with various amounts of ergosterol for 7-days, microsomes prepared and examined for their effect on Δ^7-SR activity. Fig. 6 shows that Δ^7-SR activity of the 0.5% ergosterol-fed group (S.A.=1.0 nmol/min/mg) was induced more than 2.6-fold over that of the normal-diet group (S.A.=0.38 nmol/min/mg protein). Other feeding conditions such as 0.1% and 1% ergosterol resulted in less induction of the enzyme. The peak activity of Δ^7-SR was consistently observed around 7-days. A longer period of feeding ergosterol to rats, such as 10 or 14-days, did not appear to improve the induction level of enzyme activity (data not shown). There were no particular changes in the level of endogenous hepatic microsomal cholesterol in these ergosterol-fed groups (data not shown). No detectable ergosterol was measured by GLC in this condition.

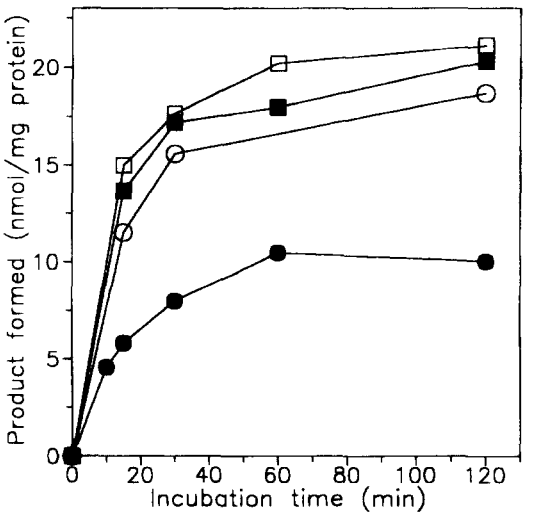

Fig. 6. Effect of feeding ergosterol on sterol Δ^7-reductase activity *in vivo*. Enzyme activity of sterol Δ^7-reductase in isolated liver microsomes from rats fed a diet supplemented with control (●), 0.1% ergosterol (○), 0.5% ergosterol (□), and 1.0% ergosterol (●), respectively, for 7-days. Each value represents the mean of duplicate determinations.

Discussion

The new assay for Δ^7-SR described here is faster, safer, and much easier than the one previously reported (Dempsey *et al.*, 1964; Dempsey, 1965; Dempsey, 1969), which mainly depended on commercially nonavailable radiolabeled sterol substrates for estimation of activity. With this assay developed for optimal conditions there is no longer requirement for another subcellular component, such as S105 fractions, and longer incubation times (Dempsey *et al.*, 1964; Dempsey, 1965; Dempsey, 1969). Although HPLC analysis of 7-dehydrocholesterol and cholesterol which are sterol components of the 7-reduction has been previously reported (Goh *et al.*, 1989), it is clear that use of ergosterol as enzyme substrate seems much easier and therefore more suitable for simple GLC analysis. Furthermore, use of ergosterol (as substrate) certainly offers many advantages over that of the radioisotope (e.g., ^{14}C-or ^{3}H-) labeled 7-dehydrocholesterol: its superior resolution from other components of enzymic reaction (i.e., microsomal endogenous cholesterol and 7-dihydroergosterol), extended detection limit (up to 1.0%

Table 2. Comparison of AY-9944-sensitive cholesterogenic enzymes with respect to inhibition by AY-9944 and induction by the CL-diet feeding

Enzyme	Substrate (sterol)	IC_{50} (μM)	Mode of Inhibition[a]	Induction by CL-diet
sterol Δ^7-reductase	ergosterol	0.25	NC	6.0
sterol Δ^8-isomerase[b]	zymosterol	1.40	M	4.0
sterol Δ^{14}-reductase[c]	4.4-dimethyl-5α-cholesta-7.14-dien-3β-ol	0.30	C	11.0

[a]NC: noncompetitive. C: competitive. M: Mixed
[b]Paik *et al.*. 1986: Kang *et al.*. 1995
[c]Kim *et al.*. 1995

conversion rate of ergosterol in GLC analysis). and convenience (e.g.. commercial availability and no need for an internal standard for quantitation of the reaction products). Despite availability of a quantitation method for 7-dehydrocholesterol in patients of SLOS (Irons *et al.*. 1994: Kelley. 1995). detailed enzymic studies of tissues or cells had relied only on radiolabeled sterol substrate during the course of that study. Hence. this new assay could be very convenient not only to review the systematic studies of the SLOS. but also to verify resolution of the Δ^7-SR in question from other cholesterogenic enzymes when proteins are contained in the same soluble state during enzyme purification.

The significance and mode of enzyme induction by feeding ergosterol. which seems to be specific for Δ^7-SR among the members of ASCE. is currently not known. Mammalian Δ^7-SR has been known to be inhibited by several cholesterol-lowering agents including AY-9944 (Dvornik and Hill. 1968). 4[2-[4-(4-chlorocinnanyl)piperazine-1-yl]ethyl]benzoic acid (BM15.766) (Pill *et al.*. 1990: Aufenanger *et al.*. 1986). and RPR101821 (Amin *et al.*. 1996). In particular. the mechanism of action as well as inhibition potencies (IC_{50}. Ki) of AY-9944 have not been reported previously. Availability of inhibition data on AY-9944 for Δ^7-SR (K_i=0.109 μM. IC_{50}=0.25 μM) as well as other members of ASCE allowed us to compare its relative potencies and mode of action against them (Table 2). Among the members of ASCE. the 7-reductase seems to be the most sensitive to AY-9944 which acts in a noncompetitive manner (Table 2). For the mode of inhibition against three members of ASCE. AY-9944 appears to act differently: Δ^7-SR. noncompetitive: Δ^{14}-SR. competitive: Δ^8-SI. mixed. Relative induction levels of ASCE by the CL-diet feeding indicate that there is apparently no correlation between degree of a diet-mediated enzyme induction and that of inhibition by AY-9944.

The fact that lathosterol is the poorest substrate for enzymic activity implies that Δ^7-SR activity does not appear to be much influenced by side-chain unsaturation or methylation in the case of the reduction of Δ^{14}C-labeled $\Delta^{5,7,24}$-cholestatrienol (Koroly and Dempsey. 1981). It is suggested that the location of Δ^7-reduction of sterol intermediate seems to fit to the terminal point of the Δ^{19}-step lanosterol transformation to cholesterol. since Δ^{24} reduction has been found to occur right after $\Delta^{8\text{-}7}$ isomerization. based on the results of substrate specificities on Δ^{24}-SR (Bae & Paik. 1997). Δ^7-SR seems to be very hydrophobic because of its precipitation nature by PEG. Although the enrichment-fold by PEG precipitation was not significantly improved over the enzyme activity in S-1. it may provide a suitable preparation for further purification of this enzyme.

In conclusion. this result should add new information to our understanding of the regulation and resolution of Δ^7-SR from this complex microsomal enzyme system that is involved in cholesterol biosynthesis from lanosterol.

Acknowledgements

We are grateful to the following people for their generous supply of the various cholesterol lowering drugs as indicated in parenthesis. Dr. Young-Ki Sim. Choongwae Pharmaceutical Co. (Lovastatin®): Mr. D. Kim. LG Chem. (cholestyramine): Dr. D. Dvornick. Wyeth-Ayerst (AY-9944): Dr. R. J. Cenedella. Kirksville College of Osteopathic Medicine. Kirksville. MO (U 18666A): Dr. B. M. Bain. Glaxo Research & Development Ltd.. Middlesex. UK (Squalestatin 1): Dr. H. W. Bohme at Marion Merrel Dow Research Institute. Cincinnati. OH (Triparanol).

This work was supported in part by the grants from the MOST (G7 to YKP) and KOSEF (9514-0401-00-12-3 to YKP) through BRC at Yonsei university.

References

Alley. T. L.. Gray. B. A.. Lee. H. S.. Scherer. S. W.. Tsui. L. C. Tint. G. S.. Williams. C. A.. Zori. R. and Wallance. M. R. (1995) *Am. J. Hum. Genet.* **56**. 1411.

Amin. D.. Rutledge. R. Z.. Needle. S. J.. Hele. D. J.. Neuenswander. K.. Bush. R. C.. Bilder. G. E. and Perrone. M. H. (1996) *Nau-Schmi. Arch. Pharmacol.* **353**. 233.

Aufenanger. J.. Pill. J.. Schmidt. F. H. and Stegmeier. K. (1986) *Biochem. Pharmacol.* **35**. 911.

Avigan. J.. Goodman. D. S. and Steinberg. D. (1963) *J. Biol.*

Chem. **236**, 1283.
Bae, S.-H. and Paik, Y.-K. (1997) *Biochem. J.* **236**, 609.
Baxter, A., Fitzgerald, B. J., Huston, J. L., McCarthy, A. D., Motteram, J. M., Ross, B. C., Sapra, M., Snowden, M. A., Watson, N. S., Williams, R. J., & Wright, C. (1992) *J. Biol. Chem.* **267**, 11705.
Dempsey, M. E. (1965) *J. Biol. Chem.* **240**, 4176.
Dempsey, M. E. (1969) *Methods Enzymol.* **15**, 501.
Dempsey, M. E., Seaton, J. D., Schroepfer, G. J., Jr. and Trockman, R. W. (1964) *J. Biol. Chem.* **239**, 1381.
Dvornik, D. and Hill, P. (1968) *J. Lipid Res.* **9**, 587.
Gaylor, J. L. (1981) (Porter, J. W., and Springer, S. L., Eds) Vol. 1, pp. 482-543, John Wiley and Sons, New York
Goh, E. H., Colles, S. M. and Otte, K. D. (1989) *Lipids.* **24**, 652.
Honda, A., Tint, G. S., Salen, G., Batta, A. K., Chen, T. S. and Shefer, S. (1995) *J. Lipid Res.* **36**, 1595.
Horie, M., Tsuchiya, Y., Hayashi, M., Iida, Y., Iwasawa, Y., Nagata, Y., Sawasaki, Y., Fukuzumi, H., Kitani, K., & Kamei, T. (1990) *J. Biol. Chem.* **265**, 18075.
Irons, M., Elias, E. R., Tint, G. S., Salen, G., Frieden, R., Buie, T. M. and Ampola, M. (1994) *Am. J. Med. Genet.* **50**, 347.
Kandutsh, A. A. (1962) *J. Biol. Chem.* **237**, 358.
Kang, M. K., Kim, C. K. Johng T. N. and Paik, Y. K. (1995) *J. Biochem.* **117**, 819.
Kelley, R. I. (1995) *Clin. Chim. Acta* **236**, 45.
Kim, C. K., Jeon, K. I., Lim, D. M., Johng, T. N., Trzaskos, J. M., Gaylor, J. L. and Paik, Y.-K. (1995) *Biochim. Biophys. Acta.* **1259**, 39.
Koroly, M. J. and Dempsey, M. E. (1981) *Lipids* **16**, 755.
Lecain, E., Chenivesse, X., Spagnoli, R. and Pompon, D. (1996) *J. Biol. Chem.* **271**, 10866.
Lowry, O. H., Rousebough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
Paik, Y.-K., Billheimer, J. T., Magolda, R. L. and Gaylor, J. L. (1986) *J. Biol. Chem.* **261**, 6470.
Paik, Y.-K., Trzaskos, J. M., Shafiee, A. and Gaylor, J. L. (1984) *J. Biol. Chem.* **259**, 13413.
Pill, J., Witte, E. C. and Schmidt, F. H. (1990) *Nau-Schmi. Arch. Pharmacol.* **341**, 552.
Rahier, A. and Taton, M. (1996) *Biochemistry* **35**, 7069.
Ritter, M. C. and Dempsey, M. E. (1970) *Biochem. Biophys. Res. Commun.* **38**, 921.
Shafiee, A., Trzaskos, J. M., Paik, Y.-K. and Gaylor, J. L. (1986) *J. Lipids Res.* **27**, 1.
Smith, D. W., Lemli, L. and Opitz, J. M. (1964) *J. Pediatr.* **64**, 210.
Tint, G. S., Irons, M., Elias, E. R., Batta, A. K., Feieden, R., Chen, T. S. and Salen, G. (1994) *Engl. J. Med.* **330**, 107.
Tint, G. S., Seller, M., Hughes-Benzie, R., Batta, A. K., Shefer, S., Genest, D. Irons, M., Elias, E. and Salen, G. (1995) *J. Lipid Res.* **36**, 89.
Trzaskos, J. M. and Gaylor, J. L. (1985) (Martonosi, A. W., Ed) Vol. 2, pp. 177-201, Plenum Press, New York
Wilton, D. C., Munday, K. A., Skinner, S. J. M. and Akhtar, M. (1968) *Biochem. J.* **106**, 803.
Wolf, C., Chevy, F., Pham, J., Kolf-Clauw, M., Citadelle, D., Mulliez, N. and Roux, C. (1996) *J. Lipid Res.* **37**, 1325.