# Enzyme activity assay for cholesterol 27-hydroxylase in mitochondria

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**Abstract Mitochondrial cholesterol 27-hydroxylase (CYP27A1) plays an important role in the maintenance of intracellular cholesterol homeostasis. Cholesterol delivery to the mitochondrial inner membrane is believed to be a rate-limiting step for the "acidic" pathway of bile acid synthesis. This work reports that proteinase K treatment of mitochondria markedly increases CYP27A1 specific activity. With endogenous mitochondrial cholesterol, treatment with proteinase K increased CYP27A1 specific activity by 5-fold. Moreover, the addition of the exogenous cholesterol in β-cyclodextrin plus proteinase K treatment increased the specific activity by 7-fold. Kinetic studies showed that the increased activity was time-, proteinase K-, and substrate concentration-dependent. Proteinase K treatment decreased the apparent $K_m$ of CYP27A1 for cholesterol from 400 to 150 μM. Using this new assay, we found that during rat hepatocyte preparation and cell culture, mitochondria gradually lose CYP27A1 activity compared with mitochondria freshly isolated from rat liver tissue.**—Li, X., P. Hylemon, W. M. Pandak, and S. Ren. **Enzyme activity assay for cholesterol 27-hydroxylase in mitochondria.** ***J. Lipid Res.*** **2006.** 47: **1507–1512.**

**Supplementary key words** cholesterol metabolism • β-cyclodextrin • proteinase K • proteolysis

Mitochondrial cholesterol 27-hydroxylase (CYP27A1; EC 1.14.13.15) is a multifunctional P450 enzyme that catalyzes the initial reaction, 27-hydroxylation of cholesterol in the "acidic" pathway of bile acid biosynthesis, and 25-hydroxylation of vitamin D3 (1). Recent data suggest that CYP27A1 plays several important roles in cholesterol homeostasis and affects atherogenesis (2). A novel mechanism described for the elimination of cholesterol from human lung macrophages and cells in arterial endothelium involves 27-hydroxylation of cholesterol by CYP27A1 (3). Patients with a rare inherited lipid storage disease, cerebrotendinous xanthomatosis, have a sterol 27-hydroxylase deficiency attributable to point mutations in the CYP27A1 gene. Manifestations of this genetically determined CYP27A1 deficiency range from accelerated atherosclerosis to progressive neurological impairment. The major symptoms in cerebrotendinous xanthomatosis are caused by the generalized accumulation of cholesterol and cholestanol in almost every tissue, including the nervous system (4, 5).

The CYP27A1 products 27-hydroxycholesterol (27-OH cholesterol) and 3β-OH-5-cholestenoic acid have been shown to function as regulatory molecules in the maintenance of intracellular cholesterol homeostasis (6–8). The potential importance of the "acidic" pathway of bile acid biosynthesis was further emphasized recently as a result of studies in *Cyp7a1*$^{-/-}$ mice (9). Despite the elimination of what is believed to be the predominant pathway of bile acid biosynthesis, a fraction of the offspring (~15%) upregulate the acidic pathway of bile acid synthesis and attain a normal life expectancy (10). Interestingly, in hepatocytes, overexpression of the gene encoding CYP27A1 led to an increase of bile acid synthesis of only 1.5-fold, compared with overexpression of the gene encoding CYP7A1, which has been shown to increase bile acid biosynthesis by 7-fold (11). These findings suggested that CYP27A1 activity must be limited by the availability of cholesterol substrate and that other mechanisms must be involved in the regulation of the "acidic" pathway of bile acid synthesis.

Recently, we found that selective overexpression of a mitochondrial cholesterol delivery protein, steroidogenic acute regulation protein, dramatically increased bile acid synthesis in vitro and in vivo (12, 13). These results raised the question of how to optimally determine CYP27A1 activity. In searching for a better enzyme assay for CYP27A1 activity, we found that treatment with proteinase K dramatically increased the specific activity in isolated rat hepatocyte mitochondria.

## MATERIALS AND METHODS

### Materials

25-Hydroxycholesterol (25-OH cholesterol) and 27-OH cholesterol were purchased from Research Plus, Inc. (Baynone, NJ).

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*Streptomyces* AP cholesterol oxidase was from Calbiochem (La Jolla, CA), and β-cyclodextrin (β-CD) was from Cyclodextrin Technologies Development, Inc. (Gainesville, FL). Proteinase K (EC 3.4.21.64) was from Sigma-Aldrich (St. Louis, MO). Cholesterol (1.5 mg/ml) was dissolved in 45% β-CD by stirring at room temperature for 24 h. All other reagents were from Sigma-Aldrich, unless indicated otherwise.

### Culture of primary rat hepatocytes

Primary male rat hepatocytes were prepared as described previously (14). Cells were plated on 150 mm tissue culture dishes (~$2.5 \times 10^7$ cells) in Williams' E medium containing dexamethasone (0.1 μM). Cells were maintained in the absence of thyroid hormone. Twenty-four hours after plating, culture medium was removed, and 20 ml of fresh medium was added. The cells were harvested for the preparation of mitochondria at the times indicated in the text.

### Preparation of mitochondrial fractions from rat liver tissue and hepatocytes

Mitochondria were isolated essentially as described previously (14). Briefly, Sprague-Dawley female rats (weighing 200–250 g) were euthanized, and liver tissue was excised. The excised liver or primary rat hepatocytes were homogenized in buffer (0.25 M sucrose, 0.5 mM EDTA, and 10 mM potassium phosphate, pH 7.4) as described previously (14). The homogenates were centrifuged at 600 *g* at 4°C for 15 min. The supernatant was then centrifuged at 6,700 *g* at 4°C for 20 min, and the pellets (mitochondrial fraction) were twice washed with the homogenization buffer. Protein concentration was determined by the Bradford dye reagent method (Bio-Rad). Freshly isolated mitochondria were used for the determination of CYP27A1 activity, because freeze-thaw was found to result in loss of activity.

### Enzyme assay for CYP27A1

Mitochondrial CYP27A1 activity was measured in a total volume of 500 μl containing 40 nmol of cholesterol dissolved in 10 μl of β-CD (45% in water), 500 μg of mitochondrial protein, 100 mM sodium phosphate, pH 7.5, 0.2 mM EDTA, 1 mM DTT, 5.0 mM trisodium isocitrate, and 0.2 units of isocitrate dehydrogenase. Reactions were initiated by adding 60 μl of 10 mM β-NADPH and incubating with shaking at 37°C for 90 min. The reactions were stopped by adding 40 μl of 40% sodium cholate. Blanks were prepared by adding sodium cholate before adding mitochondrial solution. In studies of the effects of proteinase K on CYP27A1 activity, 36 U/ml proteinase K was added to the reaction mixture. After stopping the reaction, 1.5 μg of testosterone was added to the reaction mixture as an internal standard. The sterol products were incubated with 2 units of cholesterol oxidase at 37°C for 20 min. The oxidation reaction was terminated by adding 1.5 ml of methanol followed by 0.5 ml of saturated KCl. The sterols were extracted twice using 3 ml of hexane. The hexane phase was collected and evaporated under a stream of nitrogen. The residues were dissolved in mobile phase solvents for HPLC analysis as described previously (15).

### HPLC analysis of the synthesized products

The sterol products synthesized by CYP27A1 were analyzed by HPLC on an Ultrasphere Silica column (5 μm × 4.6 mm × 25 cm; Beckman) using the HP Series 1100 solvent delivery system (Hewlett-Packard) at a flow rate of 1.3 ml/min. The chromatograph was run in a solvent system of hexane-isopropanol-glacial acetic acid (965:25:10, v/v/v) as the mobile phase. The elution profiles were monitored at 240 nm. The column was calibrated with cholesterol, 25-OH cholesterol, testosterone, and 27-OH cholesterol as described previously (15).

### Statistics

Data are reported as means ± SD. Where indicated, data were subjected to *t*-test analysis and determined to be significantly different at $P < 0.05$.

## RESULTS

After incubation of mitochondria at 37°C for 90 min in the presence or absence of β-CD, sterol products were extracted by chloroform/methanol and analyzed by HPLC as described in Materials and Methods. Isolated mitochondria contained ~50 nmol (20 μg) of mitochondrial cholesterol per milligram of protein. As shown in **Fig. 1**, β-CD significantly increased (~10-fold) 27-hydroxylation of the endogenous cholesterol (Fig. 1B). This result is consistent with a previous report of the effect of β-CD on CYP27A1 activity (16). Interestingly, the addition of proteinase K further increased the rate of 27-hydroxylation by 5-fold ($P < 0.001$) (Fig. 1C) over β-CD alone (Fig. 1B). Moreover, treatment of mitochondria with proteinase K allowed for the detection of 25-OH cholesterol formation, which is believed to be synthesized by CYP27A1 (Fig. 1C).

The effects of proteinase K treatment and β-CD-cholesterol on CYP27A1 activity were determined in mitochondria isolated from rat liver tissue, as shown in Fig. 1D–F. In the absence of β-CD-cholesterol, proteinase K did not significantly increase CYP27A1 activity (Fig. 1D) compared with control mitochondria (Fig. 1A). However, the addition of β-CD-cholesterol increased the reaction by 1.5-fold (Fig. 1E) compared with the addition of β-CD alone (Fig. 1B). Surprisingly, proteinase K treatment and β-CD-cholesterol further increased CYP27A1 specific activity by 7-fold ($P < 0.001$) (Fig. 1F) and by 3.5-fold compared with proteinase K treatment and β-CD (Fig. 1C). However, in the presence of cholesterol dissolved in ethanol, proteinase K treatment did not change CYP27A1 activity (data not shown). The effects of endogenous and exogenous cholesterol on CYP27A1 activity (**Fig. 2**) suggest that proteinase K treatment may facilitate β-CD-cholesterol complex delivery to CYP27A1.

To study the mechanism whereby proteinase K treatment increases CYP27A1 activity, a kinetic study was performed. As shown in **Fig. 3A**, in the presence of proteinase K and 80 μM β-CD-cholesterol, the rate of formation of 27-OH cholesterol was linear for at least 120 min. In contrast, without proteinase K treatment, the formation was linear for only 20 min. At 120 min, the level of 27-OH cholesterol formed was ~10-fold higher in the reaction mixture with proteinase K ($n = 5$; $P < 0.001$).

The effect of proteinase K concentration on CYP27A1 activity is shown in Fig. 3B. The increase of cholesterol 27-hydroxylation by proteinase K treatment was dose-dependent. The reaction reached a plateau at 80 units of

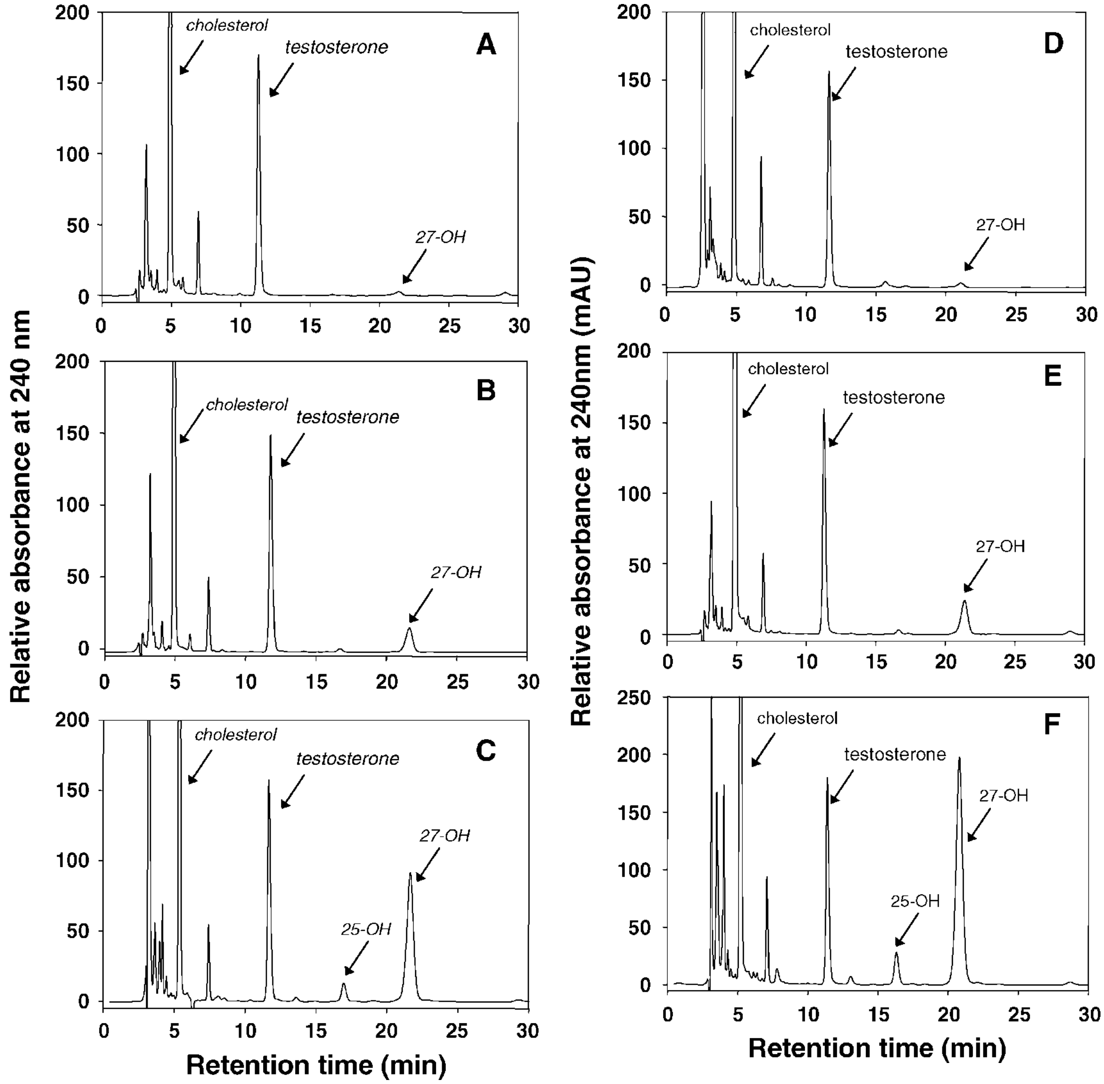


**Fig. 1.** Effects of proteinase K treatment on cholesterol 27-hydroxylase (CYP27A1) activity using endogenous and exogenous cholesterol in the isolated rat liver mitochondria. Sterol metabolites were extracted from the mitochondrial CYP27A1 activity assays as described in detail in Materials and Methods. α,β-Unsaturated ketones generated by incubating the metabolites with cholesterol oxidase were analyzed by normal-phase HPLC. A: Metabolites were extracted from control mitochondria [without β-cyclodextrin (β-CD) and proteinase K treatment]. B: Mitochondria treated with β-CD (0.9%) alone. C: Mitochondria treated with β-CD and proteinase K. D: Sterol metabolites were extracted from control mitochondria that were treated with proteinase K (without β-CD-cholesterol). E: Mitochondria treated with β-CD-cholesterol (80 μM) without proteinase K. F: Mitochondria treated with β-CD-cholesterol and proteinase K. The indicated peaks are the ketones derived from cholesterol, 25-hydroxycholesterol (25-OH), and 27-hydroxycholesterol (27-OH); testosterone propionate was used as an internal standard to monitor sterol extraction. The data represent typical results from one of three independent experiments.

proteinase K per milliliter after incubation at 37°C for 90 min. The effects of cholesterol saturation on enzymatic activity showed that the proteinase K treatment did not change $V_{max}$ (1.3 nmol/mg/min) (**Fig. 4**). In contrast, treatment of proteinase K decreased the apparent $K_m$ of CYP27A1 for cholesterol from 400 to 150 μM.

### Comparison of CYP27A1 activities in mitochondria isolated from fresh liver tissue and freshly prepared and cultured rat primary hepatocytes

To test the application of this new method for the determination of CYP27A1 activity, the specific activities of CYP27A1 in mitochondrial fractions isolated from male rat liver tissues and freshly prepared and cultured primary rat hepatocytes were determined (**Fig. 5**). The highest level of specific activity was found in the freshly isolated mitochondria from liver tissue. The measured specific activity was 20 nmol/mg mitochondrial protein in proteinase K-treated mitochondria versus 3 nmol/mg in untreated mitochondria isolated from fresh liver tissues. Without proteinase K treatment, the measured specific activities showed only a 25% decrease in freshly prepared hepatocytes and a 50% decrease in cultured primary rat hepatocytes compared with that in mitochondria from fresh liver tissue (Fig. 5A). Interestingly, with this assay, a much greater decrease in CYP27A1 specific activity was measured during

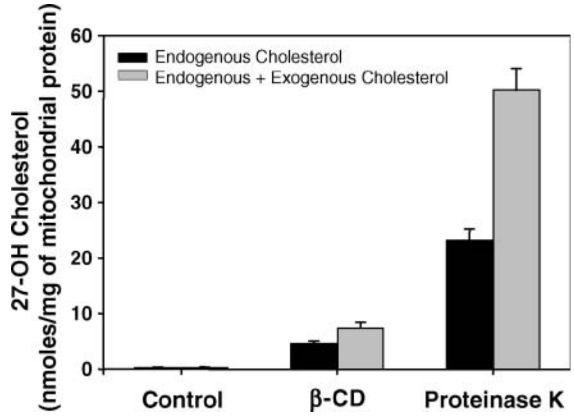


**Fig. 2.** Effects of proteinase K treatment of rat liver mitochondria on CYP27A1 activity using endogenous and exogenous cholesterol. After HPLC analysis, the total peak area of the product peak of 27-OH cholesterol was normalized to testosterone and quantitated. Each bar represents the mean ± SD (n = 5).

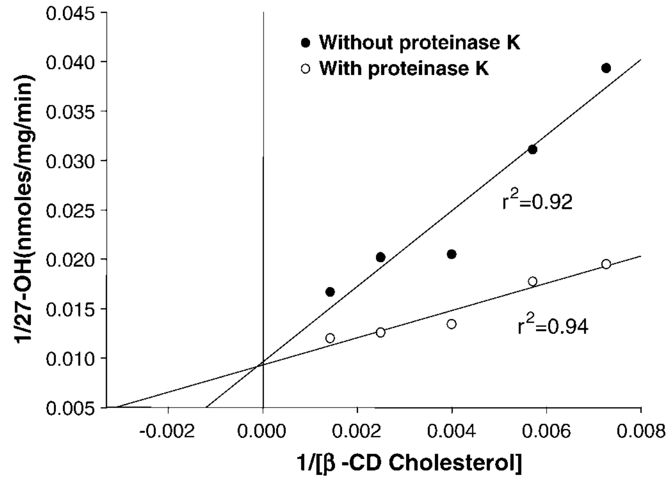


**Fig. 4.** Effect of substrate concentration on CYP27A1 kinetics in isolated mitochondria with (open circles) or without (closed circles) proteinase K treatment. The data were analyzed by Lineweaver-Burk plot. The reaction mixtures were incubated at 37°C for 20 min as described in Materials and Methods. The cholesterol concentration was varied between 37.5 and 600 μM. The data points are averages of duplicate experiments.

hepatocyte preparation and culture (Fig. 5B). The activity was decreased to 55% in the freshly prepared hepatocytes ($P < 0.01$; n = 3), and only 16% activity remained after 24 h of culture ($P < 0.01$; n = 3) compared with the mitochondria isolated from fresh liver tissue. These results indicated that the new method is much more sensitive to CYP27A1 activity changes. The mechanism of the downregulation of CYP27A1 activity is unclear at present.

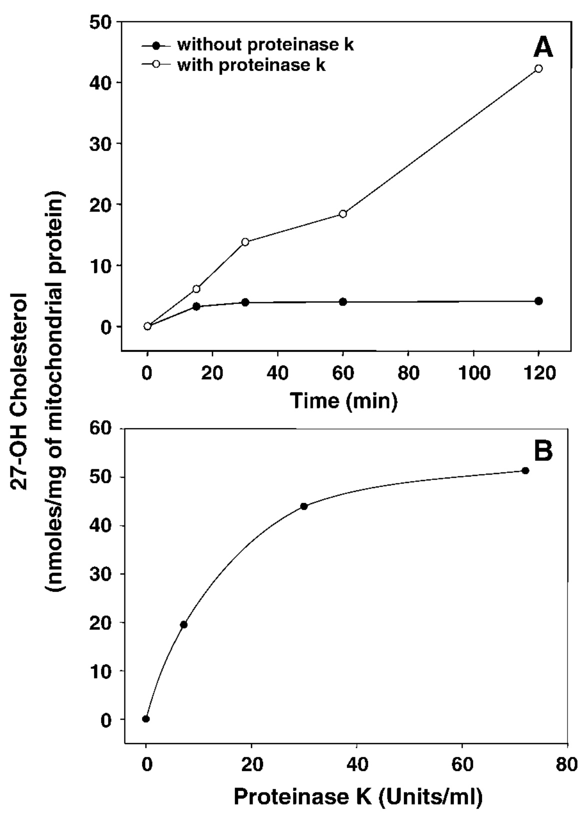


**Fig. 3.** Effect of proteinase K treatment on the rate of formation of 27-OH cholesterol. A: Five hundred micrograms of mitochondrial protein and 40 nmol of β-CD-cholesterol were incubated for various times with (open circles) or without (closed circles) proteinase K (36 U/mg). B: The concentration of proteinase K was varied in the reaction containing 500 μg of mitochondrial protein and 40 nmol of β-CD-cholesterol. The reactions were incubated at 37°C for 90 min.

## DISCUSSION

This study shows that treatment of mitochondria with proteinase K leads to a marked increase in the rates of formation of 27-OH cholesterol in isolated mitochondria. It has been reported that cholesterol added in acetone, liposomes, or detergent dispersions increases the activity of other enzymes using cholesterol as substrate, such as cholesterol 7α-hydroxylase (17) and acyl-CoA:cholesterol acyltransferase (18). However, such preparations either inhibited or only slightly enhanced CYP27A1 activity in mitochondria (16). β-CD has been used to deliver hydrophobic drugs to target sites both in vitro and in vivo; it forms water-soluble inclusion complexes with many hydrophobic drugs, including bile acids and sterols (19, 20). The addition of β-CD-cholesterol to the mitochondrial assay raised the rates of 27-OH cholesterol synthesis by >10-fold, and β-CD alone increased the enzyme activity, but to a lesser extent (16). This study shows that in the presence of β-CD-cholesterol, the treatment of mitochondria with proteinase K further increases cholesterol 27-hydroxylation by 7-fold (Fig. 1).

Several laboratories have reported that treatment of mitochondria with different proteases inactivated the import of in vitro-synthesized mitochondrial precursor proteins into these organelles (21, 22). Treatment of isolated yeast mitochondria with high levels (1 mg/ml) of trypsin severely inhibits protein import but does not destroy the integrity of the outer membrane or abolish mitochondrial energy coupling (22). Thus, it is unlikely that proteinase K

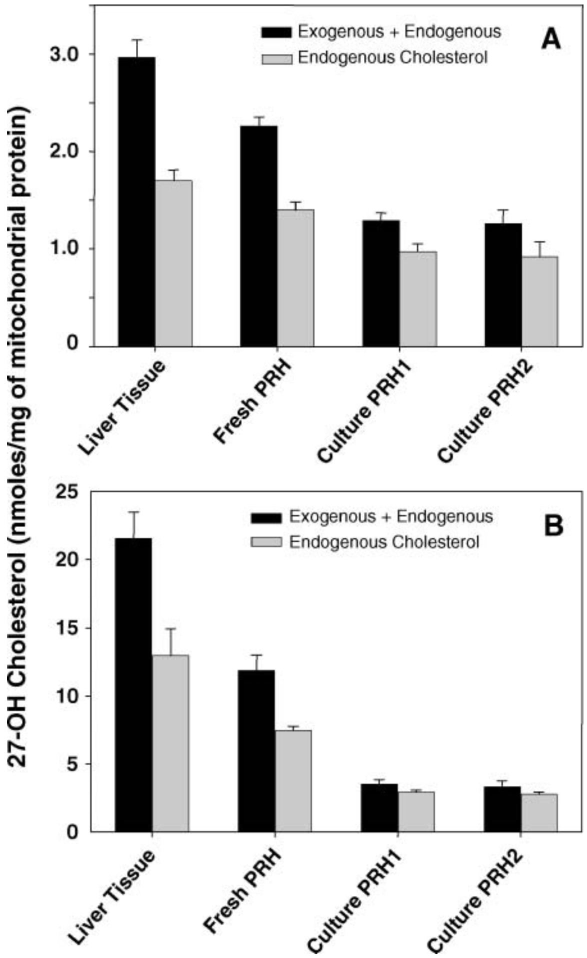


**Fig. 5.** CYP27A1 activity in mitochondria isolated from fresh rat liver tissue (Liver Tissue), freshly prepared rat hepatocytes (Fresh PRH), and primary rat hepatocytes cultured for 24 h (Culture PRH1) and for 48 h (Culture PRH2). Mitochondria were isolated as described in Materials and Methods. The activities in 500 μg of mitochondrial protein were assayed with either endogenous cholesterol, 0.9% β-CD alone (gray bars) or with 40 nmol of exogenous cholesterol in 0.9% β-CD (black bars). A: Without proteinase K treatment. B: With proteinase K treatment. The data represent means ± SD (n = 3).

treatment could activate CYP27A1 activity directly. However, it is possible that treatment of mitochondria with proteinase K may open channels in the outer mitochondrial membrane that facilitate β-CD-cholesterol access to the inner membrane of mitochondria, where CYP27A1 is located. In our experiments, proteinase K treatment alone did not increase CYP27A1 specific activity (Fig. 1A) and proteinase K treatment plus β-CD-cholesterol decreased its apparent $K_m$ (Fig. 4), supporting this hypothesis. It is also possible that treatment of proteinase K could increase the efflux of products such as 25- and 27-OH cholesterols, which can serve as competitive substrates for further oxidation. However, this study did not address that issue.

The increase in CYP27A1 specific activity by treatment with proteinase K was dose- and time-dependent. The method described here can be used to determine more precisely the specific activities of CYP27A1 in isolated mitochondria. Using our method, we found that CYP27A1 specific activity in mitochondria was gradually lost during the preparation and cell culture of primary hepatocytes, although the mechanism is not clear at present (Fig. 5).

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