methods

A simple and rapid method to measure cholesterol binding to P450s and other proteins

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Abstract **Cholesterol plays an important role in cellular function and membrane compartmentalization and is involved in the interaction with more than a dozen of different proteins. Using three cholesterol-metabolizing cytochrome P450s (P450s 7A1, 46A1, and 11A1), we have developed a rapid and simple assay for measurements of nanomolar to micromolar cholesterol affinities. In this assay, the P450 is incubated with a fixed amount of radiolabeled cholesterol and varying concentrations of cold cholesterol followed by separation of free and protein-bound cholesterol via filtration through a membrane. Free cholesterol is found in the flow-through fraction, whereas P450 binds to the membrane. The radioactivity of the membranes is then measured, and a saturation curve is generated after correction for nonspecific binding of cholesterol to the filter. The validity of the filter assay was confirmed by spectral assay, a traditional method to evaluate the interaction of the P450 enzymes with their substrates. Two types of membranes, one binding positively charged proteins and another binding negatively charged proteins, were identified. These membranes were also found to hold proteins through hydrophobic interactions. Thus, the cholesterol binding properties of a wide variety of proteins could be characterized using this filter assay.**—Mast, N., and I. A. Pikuleva. **A simple and rapid method to measure cholesterol binding to P450s and other proteins.** ***J. Lipid Res.*** **2005.** 46: **1561–1568.**

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Cholesterol is essential for most mammalian cells, where it is used either as a structural component of cellular membranes or as a substrate for the synthesis of steroid hormones and bile acids. Cholesterol also serves as a regulator of gene transcription, protein degradation, and enzyme activity and has been implicated in programmed cell death (1, 2). Moreover, the role of cholesterol in signal transduction is being increasingly recognized (3). A number of proteins have been shown or suggested to interact with cholesterol. They include apolipoprotein A-I (4), caveolin (5), steroidogenesis acute regulatory protein (6), hedgehog protein (7), annexin II (8), sterol carrier protein-2 (9), peripheral benzodiazepine receptor (10), cholesterol oxidase (11), cholesterol dehydrogenase (12), acyl-CoA cholesterol acyltransferase (13), Niemann-Pick type C2 protein (14), Niemann-Pick C1-like 1 protein (15), cholesterol binding cytolytic bacterial toxins (16), synaptophysin (17), the sterol-regulatory element binding protein cleavage-activating protein (18), retinoic orphan receptor α (19), cholesterol 25-hydroxylase (20), and cytochrome P450 enzymes (P450s or CYPs) 11A1, 7A1, 27A1, 46A1, and 3A4 (21–24). Several different approaches were used to evaluate the ability of these proteins to bind cholesterol. They were based on: *1*) use of a fluorescent cholesterol derivative that changes its fluorescence when bound to a protein (25); *2*) immunostaining of a protein bound to either a TLC or a microtiter plate coated with cholesterol (26, 27); *3*) detection of a spectral change in a protein induced by cholesterol binding (28–30); and *4*) radioactivity measurements after a protein was incubated with a mixture of radiolabeled and unlabeled cholesterol and separated from unbound cholesterol either via affinity or other types of classic column chromatography (18, 31) or via centrifugation of the assay mixture through microcolumns (14, 32).

This laboratory is focused on structure/function studies of four cholesterol-metabolizing P450s. Three of them play key roles in cholesterol degradation in humans by initiating the classical bile acid biosynthetic pathway in the liver (CYP7A1) and the alternative pathways of bile acid biosynthesis in extrahepatic tissues (CYP27A1) and brain (CYP46A1). The fourth P450 is CYP11A1, which catalyzes the first step in overall steroid hormone biosynthesis in steroidogenic tissues. CYP7A1, CYP27A1, CYP46A1, and CYP11A1 bind cholesterol with nanomolar to low micromolar constants (the K_d value depends on whether detergent is present and at what concentration in the assay buffer) (29, 30, 33) but metabolize it to different products: 7α-hydroxycholesterol (CYP7A1), 27-hydroxycholesterol

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Abbreviations: CYP, cytochrome P450; HPCD, 2-hydroxypropyl-β-cyclodextrin.

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(CYP27A1), 24*S*-hydroxycholesterol (CYP46A1), and pregnenolone (CYP11A1). Cholesterol-metabolizing P450s belong to the P450 superfamily of enzymes that contain an iron protoporphyrin IX as a prosthetic group (34). The distinctive feature of P450s is that unlike many other b-type hemoproteins, a thiol group from a cysteine residue of the protein serves as the fifth ligand to the heme iron and a water molecule occupies the sixth coordination position when substrate is not present in the enzyme active site (reviewed in 35). P450s are the major enzymes involved in the metabolism of numerous endogenous (cholesterol, steroid hormones, bile acids, vitamins A and D_3, fatty acids, prostaglandins, leukotrienes, and biogenic amines) and exogenous (most drugs and environmental pollutants) compounds. In many but not all P450s, substrate binding results in a displacement of the water molecule from the coordination sphere of the heme iron, leading to a change of the absorbance maximum of the P450 from 418 to 393 nm (36). This substrate-induced spectral shift is used to measure substrate binding.

Recently, we undertook a comprehensive investigation to identify amino acid residues in the CYP7A1 active site that are involved in the interaction with cholesterol (37). Forty-one mutants, encompassing 26 amino acid residues, were generated and characterized with respect to cholesterol binding and kinetic parameters for cholesterol hydroxylation. The problem that we encountered during the course of these studies was that cholesterol did not induce a spectral shift in some of the mutants as it did in the wild-type enzyme, making interpretation of the results difficult. To overcome this problem, we developed an alternative assay to measure cholesterol binding, the filter assay, which is not based on the P450 spectral response. Here, we explore the versatility of this assay by investigating whether it could be used for studies of other cholesterol-metabolizing P450s, such as CYP46A1 and CYP11A1. The three enzymes share <25% sequence identity. We also tested whether nanomolar affinities could be measured by this assay. The data obtained indicate that the filter assay can be used as an alternative or complementary method to the spectral assay to measure cholesterol binding to different P450s and possibly to other cholesterol binding proteins as well.

MATERIALS AND METHODS

Materials

Unlabeled cholesterol, cytochrome *c*, and 2-hydroxypropyl-β-cyclodextrin (HPCD) were from Sigma-Aldrich (St. Louis, MO); the nonionic detergent $C_{12}E_8$ was from Anatrace (Maumee, OH); [^{3}H]cholesterol was from Amersham Bioscience (Piscataway, NJ); I.C.E. 450 polysulfone and SB-6407 membrane disk filters (pore size, 0.45 μm; diameter, 47 mm) were from Pall Life Science (Ann Arbor, MI); Tween 20 was from J. T. Baker (Phillipsburg, NJ). Unlabeled and [^{3}H]5β-cholestane-3α,7α,12α-triol were a generous gift from Dr. I. Bjorkhem (Karolinska Institute, Huddinge, Sweden), who synthesized them as described (38). All chemicals were of American Chemical Society grade. The filter holder was composed of a glass funnel (15 ml), a spring clamp, and a fritted glass base with stopper, all from Millipore (Bedford, MA). Borosilicate culture tubes (10 mm × 75 mm) were from Corning Glass (Corning, NY). GraphPad Prism software was from GraphPad (San Diego, CA).

Recombinant truncated CYP7A1 and full-length CYP46A1 and CYP11A1 were expressed and partially purified as described (30, 39, 40). The specific heme content of these preparations was 10–17 nmol/mg protein.

Spectral assay for cholesterol binding

The assay was performed at 18°C in either 50 mM potassium phosphate buffer (PBS), pH 7.4, containing 20% glycerol and 1 mM EDTA (detergent-free buffer) or in the same buffer but containing 0.05% Tween 20 (detergent-containing buffer). The concentration of P450 was 0.5 μM. Cholesterol was added from 0.25 mM stock in 4.5% HPCD to titrate CYP7A1 and CYP11A1 in detergent-free buffer or from 10 mM stock in 45% HPCD to titrate CYP7A1 and CYP46A1 in detergent-containing buffer. The total volume of the added steroid was no more than 10 μl. The P450 sample in the assay buffer (1 ml) was placed in each of two matched cuvettes. Then, varying amounts of cholesterol (1–50 μM) were added to the sample cuvette. After each addition of cholesterol, the solution was mixed and spectral measurements were taken with a 1 min interval until no further increase in $\Delta A_{390\text{-}420}$ was observed (usually it takes 3–5 min for the full spectral response to develop). GraphPad Prism software was used to calculate apparent binding constants (K_d) by fitting spectral data into one of the following equations:

$$\Delta A = (\Delta A_{max}[S])/(K_d + [S])$$

or

$$\Delta A = 0.5\Delta A_{max}(K_d + [E] + [S] - \sqrt{(K_d + [E] + [S])^2 - 4[E][S]})$$

where ΔA is the spectral response at different substrate concentrations [S], ΔA_{max} is the maximal amplitude of the spectral response, and [E] is the enzyme concentration. The former equation was applied when K_d was higher than the enzyme concentration, and the latter when K_d was similar to or less than the enzyme concentration, assuming 1:1 stoichiometry. After each experiment, the P450 content was quantified by the CO difference spectrum of the reduced form of the enzyme (34) to confirm that there was no denaturation during titration.

Filter assay for cholesterol binding

The assay was conducted in small borosilicate culture tubes with the total volume of the assay mixture being 50 μl. Each tube contained the assay buffer, 0.5–1 μM P450, 45 nM [^{3}H]cholesterol (100,000 cpm), and a range of concentrations (0–50 μM) of cold cholesterol. Reagents were mixed in the assay buffer in the following order: radioactive cholesterol, P450, and cold cholesterol. When detergent-containing buffer was used, radioactive and cold cholesterol were added from 900 nM and 1 mM stock solutions, respectively, in 50 mM PBS, pH 7.4, 20% glycerol, 4.5% HPCD, 0.05% Tween 20, and 1 mM EDTA. When detergent-free buffer was used, stock solutions of radioactive cholesterol (900 nM) and cold cholesterol (0.025 mM) were prepared using 50 mM PBS, pH 7.4, 20% glycerol, 0.9% HPCD, and 1 mM EDTA. The total volume of the solution from which cholesterol stock was added was then adjusted to 5 μl to have the same concentration of HPCD in each tube. Control incubations with all of the components, except P450, were also prepared for each point in the saturation curve. Tubes were kept on ice for 30 min, and then the content of each tube was filtered onto a separate premoistened membrane placed in the filter holder. Filters were washed with 0.5 ml of the assay buffer, dried for 3 h at 37°C, and

placed in 5 ml of scintillation liquid, and the radioactivity was counted.

Determination of apparent binding constants of cholesterol for HPCD and Tween 20

The K_d measurements were based on an approach used by others (41), in which the turbidity of solutions containing a suspension of a hydrophobic compound is decreased in the presence of increasing concentrations of HPCD. Using this approach, we determined the affinity of cholesterol for HPCD in detergent-free buffer (37). To accurately calculate the cholesterol affinity for HPCD in Tween-containing buffer, we needed to know the K_d value of cholesterol for Tween 20. This K_d was determined as described previously for HPCD (37). A suspension of cholesterol (0.5 mM) in 50 mM PBS, pH 7.4, containing 20% glycerol and 1 mM EDTA was prepared and divided into 20 × 0.5 ml aliquots to which different amounts of Tween (0–8%) were added from 10% stock. Both a suspension of cholesterol and a stock solution of Tween 20 were prepared well in advance and maintained at room temperature for a minimum of 16 h. After the addition of Tween 20 to the cholesterol suspension, aliquots were left at room temperature under constant stirring, and their optical densities (attributable to turbidity) were measured at 450 nm at 16 and 40 h after the addition of Tween; there was essentially no difference between the two time points. The optical density of an aliquot at a given concentration of Tween was multiplied by a dilution factor and plotted versus Tween concentration. The K_d was calculated with GraphPad Prism software using the following equation:

$$A = 0.5A_{max}(K_d + [C] + [Tween] - \sqrt{(K_d + [C] + [Tween])^2 - 4[C][Tween]})$$

where A is the optical density at different detergent concentrations [Tween], A_{max} is the maximal optical density, and [C] is the cholesterol concentration. To compare the K_d value of cholesterol for Tween with that for HPCD, we converted percentage concentrations of Tween into micromolar concentrations, assuming that the average molecular weight of Tween 20 is 1,228. The K_d of cholesterol for HPCD in the presence of 0.5% Tween 20 was then calculated as described by others (42), assuming competition for cholesterol between Tween and HPCD. Data were fit to the following model using the program DynaFit (43):

$$\text{Tween} + \text{cholesterol} \leftrightarrows \text{Tween cholesterol} \quad K_d^{L1}$$

$$\text{HPCD} + \text{cholesterol} \leftrightarrows \text{HPCD cholesterol} \quad K_d^{L2}$$

RESULTS

Optimization of the filter assay conditions for CYP7A1 and CYP46A1 in detergent-containing buffer using negatively charged membranes

The choice of the membrane filter was based on our knowledge obtained while developing a purification protocol for CYP7A1. We knew that this enzyme binds tightly to a resin SP-Sepharose that contains negatively charged sulfopropyl groups; therefore, a search of commercially available strongly acidic, negatively charged membrane filters was conducted. As a result, the I.C.E. 450® membrane filters from Pall Life Sciences were identified. Their ion-exchange properties are provided by the sulfonic acid active sites.

At first, we tested the capacity of these membranes to bind CYP7A1. No enzyme was detected in the flow-through fraction, as assessed by the CO-reduced difference spectrum, when 25, 50, and 75 pmol in 50 μl of the detergent-containing buffer (0.05% Tween 20) were applied to the membrane and the membrane was washed with 500 μl of the buffer. When the CYP7A1 quantities were increased to 100, 200, and 500 pmol, ~10, 14, and 40%, respectively, of the enzyme went through the filter. We also established that there is no P450 elution when the membrane is washed with the buffer.

When detergent is present in the assay mixture and cholesterol is added from a stock solution in HPCD, it is important to know the binding constants of cholesterol for the detergent and for HPCD to evaluate whether these compounds will affect the affinity of cholesterol for CYP7A1 under the assay conditions. At first, the apparent binding constants of cholesterol for Tween 20 and HPCD in 50 mM PBS containing 20% glycerol and 1 mM EDTA were determined (2.46 and 1.05 mM, respectively), and then the former was used to fit the data when cholesterol was titrated with HPCD in the presence of 0.05% Tween. The calculated K_d of cholesterol for HPCD under these conditions was 1.45 mM. From the titration curves (**Fig. 1**), we also determined that the minimal molar excess of HPCD over cholesterol to dissolve the latter should be ~130 in detergent-free buffer and 15 in the presence of 0.05% Tween 20. According to the spectral assay (**Fig. 2**), the K_d of cholesterol for CYP7A1 in detergent-containing buffer is 4.3 μM. Therefore, cholesterol concentrations up to ~50 μM should be used to build the saturation curve. Keeping that in mind, as well as the fact that we have to dilute stock solution of cholesterol at least 10-fold to stimulate cholesterol dissociation from the HPCD cavity, 4.5% (~37.5 mM) HPCD was used to prepare 1 mM cholesterol

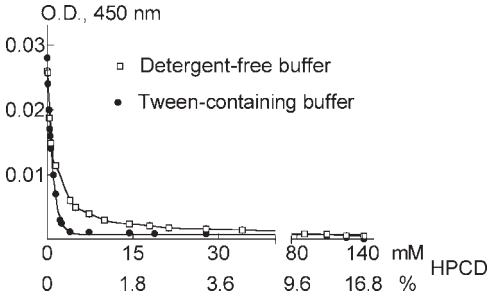

Fig. 1. Cholesterol binding to 2-hydroxypropyl-β-cyclodextrin (HPCD) in detergent-free and Tween-containing buffer. A suspension of cholesterol (0.5 mM) in either 50 mM PBS, pH 7.4, containing 20% glycerol and 1 mM EDTA or in the same buffer plus 0.05% Tween 20 was prepared and divided into 20 × 0.5 ml aliquots to which different amounts of HPCD were added from 45% stock. Aliquots were left at room temperature under constant stirring, and their optical densities (O.D.; attributable to turbidity) were measured at 450 nm at 16 and 40 h after the addition of HPCD; there was essentially no difference between the two time points. The optical density of an aliquot at a given concentration of HPCD was then multiplied by a dilution factor and plotted versus HPCD concentration. The K_d was calculated with GraphPad Prism software as described in Materials and Methods.

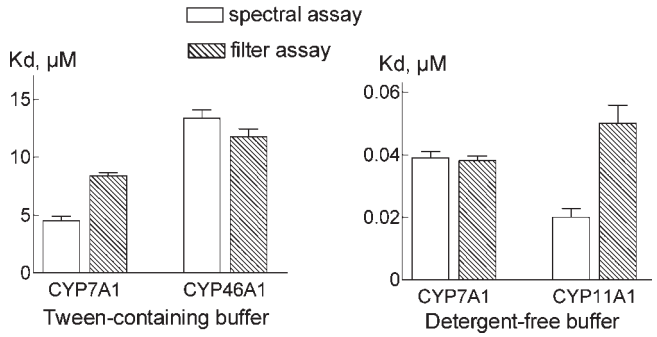

Fig. 2. Comparison of the K_d values determined by the filter and spectral assays. Assays were performed as described in Materials and Methods. The results shown are means ± SD of three independent measurements. CYP7A1, cytochrome P450 7A1; CYP46A1, cytochrome P450 46A1.

stock for the filter assay in detergent-containing buffer. Labeled cholesterol was also prepared in 4.5% HPCD. Next, we determined whether cholesterol binds to the I.C.E. membranes. Of 100,000 cpm of [^{3}H]cholesterol in 50 μl of the Tween-containing buffer that were applied to the filter, only ~6,000 cpm, or 6%, were found to bind to the membrane after it was washed with 500 μl of the buffer. We also confirmed that 500 μl of the buffer is enough to remove all unbound cholesterol.

In the fourth set of experiments, 1 μM P450 was incubated with small ([^{3}H]cholesterol only) and large ([^{3}H] cholesterol plus 50 μM cold cholesterol) amounts of the substrate, and then 25, 50, 100, 200, and 500 μl of the assay mixture containing 25, 50, 100, 200, and 500 pmol of CYP7A1, respectively, was applied to the membrane. The membrane was washed and dried, and the radioactivity was counted. A linear increase of radioactivity versus P450 concentration was observed up to 75 pmol of CYP7A1 applied to the membrane, independent of whether P450 was incubated with small or large amounts of cholesterol (data not shown).

The K_d of cholesterol for CYP7A1 was then determined. The assay mixture (50 μl) contained 50 pmol of P450, 2.2 pmol of [^{3}H]cholesterol (100,000 cpm), and different amounts (0–2,500 pmol) of cold cholesterol. To calculate the K_d, the data (the upper curve in **Fig. 3A**) were corrected for the nonspecific binding of cholesterol to the filter (binding in the absence of P450; the lower curve in Fig. 3A). The radioactivity of the filters at various concentrations of cold cholesterol was then subtracted from the radioactivity of the filter when no cold cholesterol, but only P450, was present in the assay buffer, and the resulting data were plotted versus the concentration of cold cholesterol and fit (Fig. 3A, inset) as described for the spectral binding assay in Materials and Methods. The K_d value determined by the filter assay appeared to be in the same range as that of the spectral assay (Fig. 2). Approximately 85% of radioactive cholesterol was displaced in CYP7A1 by cold cholesterol at saturating cholesterol concentrations, indicating that ~15% of this substrate was bound nonspecifically to the P450.

Three control experiments for specificity of cholesterol binding were also conducted. In the first, CYP7A1 used in the assay was replaced with the same amount of cytochrome *c* (50 pmol), a protein that is not known to bind cholesterol. In a preliminary study, we established that cytochrome *c* does bind to the I.C.E. membrane and that up to 100 pmol of the protein could be applied to the membrane without detecting it in the flow-through fraction. As seen in **Fig. 4A**, independent of whether cytochrome *c* was present or absent in the assay buffer, the same amount of radioactive cholesterol was bound to the filter, and a cholesterol binding curve was not observed. In the second control experiment, an inactive form of CYP7A1, so-called

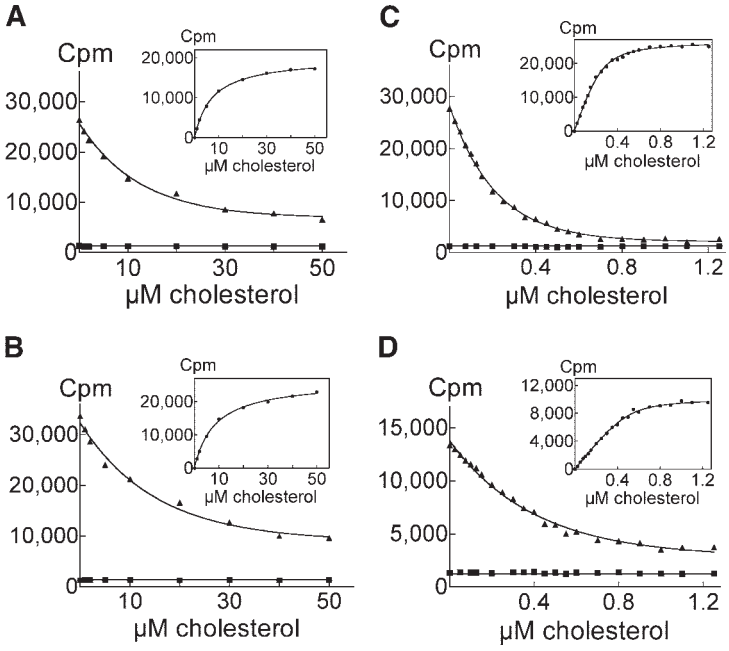

Fig. 3. Representative curves of cholesterol binding to CYP7A1 (A) and CYP46A1 (B) in the Tween-containing buffer and to CYP7A1 (C) and CYP11A1 (D) in detergent-free buffer. The inset in each panel shows the fit of the corrected data by GraphPad Prism software as described in Materials and Methods. Each data point (closed triangles) was obtained by incubating 0.5–1 μM P450 with 45 nM [^{3}H]cholesterol (100,000 cpm) and a range of concentrations (0–50 μM) of cold cholesterol in a test tube in a total volume of 50 μl of the assay buffer. Control incubations with all of the components, except P450 (closed squares), were also prepared for each point in the saturation curve. Tubes were kept on ice for 30 min, and then the content of each tube was filtered onto a separate premoistened membrane placed in the filter holder. Filters were washed with 0.5 ml of the assay buffer, dried for 3 h at 37°C, and placed in 5 ml of scintillation liquid, and the radioactivity was counted.

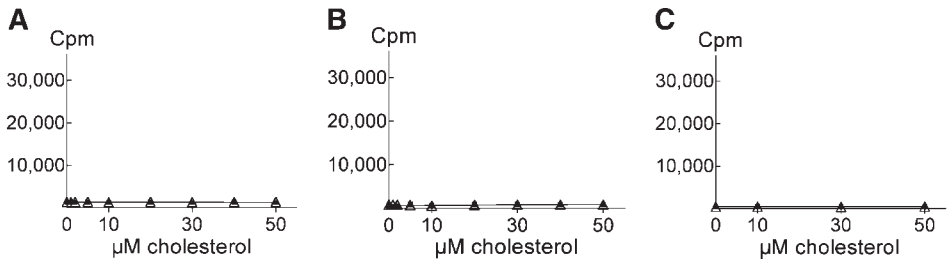

Fig. 4. Control incubations for the specificity of cholesterol binding. A: The filter assay was carried out as described for Fig. 3, except that 1 μM cytochrome *c* was used instead of CYP7A1. The assay buffer was 50 mM PBS, pH 7.4, containing 20% glycerol, 1 mM EDTA, and 0.05% Tween 20. B: An inactive form of CYP7A1, cytochrome P420, was used, and the assay buffer contained 0.05% $C_{12}E_8$ instead of 0.05% Tween 20. C: Both radioactive and cold cholesterol were replaced with radioactive and cold 5β-cholestane-3α,7α,12α-triol, and CYP7A1 was incubated with this steroid in 50 mM PBS, pH 7.4, containing 20% glycerol, 1 mM EDTA, and 0.05% Tween 20. Open triangles represent the radioactivity of the filters through which protein-containing incubations were filtered, and closed squares represent the radioactivity of the filters through which incubations containing no protein were filtered.

cytochrome P420, was used instead of the active, P450, form of the enzyme. P420 was obtained by incubating CYP7A1 at 42°C for 1 h in the presence of the nonionic detergent $C_{12}E_8$ (0.05%). As in the first control, there was no difference in the radioactivity of the membranes through which incubations with and without the P420 protein were filtered (Fig. 4B). It is interesting that the substitution of 0.05% Tween 20 with 0.05% $C_{12}E_8$ in the assay buffer led to an ~2-fold decrease in the amount of [^{3}H]cholesterol that bound nonspecifically to the filter. Finally, in the third control experiment, CYP7A1 was incubated with 5β-cholestane-3α,7α,12α-triol instead of cholesterol. 5β-Cholestane-3α,7α,12α-triol neither induces a spectral response in CYP7A1 nor is metabolized by this enzyme. Again, as in the two previous experiments, all membranes gave essentially the same radioactive count (Fig. 4C). Compared with cholesterol, much less [^{3}H]5β-cholestane-3α,7α,12α-triol (~5-fold) was found to bind nonspecifically to the membrane under identical assay conditions, likely because 5β-cholestane-3α,7α,12α-triol is a much more polar steroid than is cholesterol. Thus, the three control experiments suggest that the binding curve in Fig. 3A is specific for cholesterol.

The conditions of the filter assay for CYP46A1 were optimized using exactly the same strategy as described for CYP7A1. Cholesterol has less affinity for CYP46A1 than for CYP7A1, although its spectral K_d value still falls in the low micromolar range (Fig. 2). Therefore, 1 mM cholesterol stock in 4.5% HPCD was used. The K_d value determined by the filter assay appeared to be very similar to that determined by the spectral assay (Figs. 2, 3B). Nonspecific binding of cholesterol to CYP46A1 was 25%, which probably reflects the fact that full-length CYP46A1 is a much more hydrophobic enzyme than is the truncated form of CYP7A1 that was used in the present work.

Optimization of the filter assay conditions for CYP7A1 in detergent-free buffer

A stretch of hydrophobic residues 2–24 that anchors P450 to the membrane was removed by genetic engineering in the truncated form of CYP7A1. As a result, CYP7A1 became a soluble enzyme that does not precipitate from the solution in the absence of detergent. In our previous study (37), Tween 20 was included in the buffer for K_d determination because K_d values of the mutant P4507A1 were compared with their K_m values, and the latter were measured in the presence of 0.05% Tween 20. At this detergent concentration, a maximal, >4-fold stimulation of the enzyme activity was observed compared with that in detergent-free buffer. Tween 20, however, increased the spectral K_d of cholesterol for CYP7A1 by 100-fold (0.04 vs. 4.3 μM) (Fig. 2). We tested whether the filter assay could be used to quantitate tight cholesterol binding. Based on spectral studies, the K_d of cholesterol for CYP7A1 in the absence of Tween 20 was expected to be in the nanomolar range; consequently, 0.9% (~7.5 mM) HPCD was used to prepare 0.025 mM cholesterol stock. As in the case of the detergent-containing buffer, there was a good correspondence between the K_d values determined by the filter assay and the spectral assay (Figs. 2, 3C). Nonspecific cholesterol binding to CYP7A1 under the experimental conditions used was 8%.

Filter assay for CYP11A1 in detergent-free buffer using positively charged membranes

To test the versatility of the filter assay, we investigated whether the third cholesterol-metabolizing P450, CYP11A1, binds to the I.C.E. membranes. When 25–100 pmol of the enzyme was applied to the filter in detergent-free buffer, no protein was detected in the flow-through fraction. However, when 0.05% Tween was included in the buffer, almost all P450 went through the membrane, suggesting that this P450 is held on the filter through hydrophobic but not ionic interactions, as expected. Therefore, positively charged SB-6407 membrane filters that contain quaternary ammonium groups as the source of the charge were tested. As with negatively charged membranes, CYP11A1 binding was observed in detergent-free buffer but not in the presence of 0.05% Tween 20. Because nonspecific cholesterol binding (binding in the absence of detergent and P450) to positively charged membranes was insignifi-

cant (~6%) and similar to that observed with negatively charged membranes, we determined the K_d of CYP11A1 for cholesterol in detergent-free buffer using positively charged membranes. This value appeared to be in the same range as that determined by the spectral assay (Figs. 2, 3D). Nonspecific cholesterol binding to CYP11A1 was 28%.

DISCUSSION

In this report, we describe a simple and rapid method to measure cholesterol binding. Three P450 enzymes that share low sequence identity were used to test the validity of this method. Two of them reside in the endoplasmic reticulum (CYP7A1 and CYP46A1) and one in the inner mitochondrial membrane (CYP11A1). These enzymes are also different in terms of their hydrophobicity, which increases in the following order: truncated form of CYP7A1 < full-length CYP11A1 < full-length CYP46A1. Two types of membrane filters that do not significantly bind cholesterol were identified. One of them, I.C.E. 450, is strongly acidic and negatively charged, and another, SB-6407, is strongly basic and positively charged. Quite surprisingly, both of the filters were found to bind CYP11A1 via hydrophobic interactions likely provided by the copolymer filter media. Thus, using either of the membranes, the cholesterol binding properties of a wide variety of proteins, positively and negatively charged as well as hydrophobic, could be evaluated by the filter assay.

Aqueous solutions of HPCD were used to dissolve cholesterol. HPCD solubilizes hydrophobic compounds by forming an inclusion complex in which the solubilized lipophile is partially inserted in the hydrophobic cavity of HPCD (44). The advantage of cyclodextrin-solubilized compounds is that they do not precipitate upon dilution in aqueous medium, and in the case of moderately to weakly bound lipophiles (binding constants of 100 μM or higher), their release from the cyclodextrin inclusion complexes is essentially instantaneous and quantitative (45). The average lifetime of a lipophile in the cyclodextrin cavity was found to be in the millisecond to microsecond range or shorter, dependent on the compound with simple dilution being the major driving force for dissociation of weakly to moderately bound complexes (24). The binding constants of cholesterol for HPCD in detergent-containing and detergent-free buffers were determined as well as the minimal ratio of HPCD over cholesterol that is required to keep cholesterol in solution. The millimolar binding constants of cholesterol for HPCD in the buffers used in the present study suggest that this vehicle does not significantly affect cholesterol affinity for the P450s tested, because their affinities were in the nanomolar to low micromolar range. We compared the filter assay with the spectral assay, a traditional method to measure the substrate binding in P450s. The two methods gave very similar K_d values, verifying the validity of the filter assay.

A detailed, step-by-step description of how assay conditions were optimized is presented here to provide a paradigm for investigators who want to use this assay in their studies. Several assays of a similar type are described in literature (14, 18, 31, 32). Okamura and colleagues (14) mixed their protein of interest with increasing concentrations of radiolabeled cholesterol, followed by separation of the bound and free cholesterol by gel filtration on Probe-Quant™ G-50 Micro Columns (Pharmacia Biotech) and counting the radioactivity of the resulting eluate. This experimental design, however, does not allow one to discriminate between specific and nonspecific cholesterol binding to the protein. To evaluate whether there is nonspecific binding, the authors had to delipidate the protein by precipitating it with acetone, then resuspend the delipidated protein in the buffer, measure its cholesterol binding capacity, and compare it with that obtained using the untreated protein. Our assay is based on the displacement of radiolabeled cholesterol with increasing concentrations of cold cholesterol, which allows the quantification of both specific and nonspecific cholesterol binding. Ko and colleagues (32) also used cholesterol displacement as one of the approaches to calculate the K_d. However, they separated free and bound cholesterol by centrifugation using a CentriSep gel filtration column (Princeton Separation) and measured the radioactivity of the flow-through fraction. CentriSep gel filtration columns were found to have column-to-column variability in the amount of the protein that was nonspecifically bound. For this reason, the same column was used to separate bound and free cholesterol for each point in a saturation curve, which significantly extended the time required to determine one K_d value. There was also increased cholesterol flow-through from the column at concentrations of >300 nM (32). For the studies of Ko and colleagues (32), this was not important because they had to determine nanomolar K_d values, but it will certainly complicate the measurements of micromolar binding constants. Tsujishita and Hurley (31) and Radhakrishnan and colleagues (18) incubated their proteins with increasing concentrations of radiolabeled cholesterol, like Okamura et al. (14), but they used nickel-nitrilotriacetic acid agarose resin to isolate protein-bound cholesterol and did not test for nonspecific cholesterol binding. They also had to construct a histidine-tagged protein.

The advantages of the filter assay described in the present work can be summarized as follows: *1*) it allows the quantification of nanomolar to micromolar binding constants; *2*) it discriminates between specifically and nonspecifically bound cholesterol; *3*) it does not require protein modification by genetic engineering; and *4*) it is a relatively low-cost assay; we cut each membrane into four parts and used one quarter for each point in the saturation curve. Although our filter assay was developed to measure cholesterol binding, we believe it could be applied to any steroid, provided that the steroid does not bind to the membrane. Most of the steroid hormones are metabolized by P450 enzymes, which are now extensively studied by site-directed mutagenesis. Although the spectral assay will remain the assay of choice for P450 investigators, the filter assay could be used to characterize P450 mutants that do not exert spectral responses upon substrate binding. It will help to clarify whether the substrate indeed does not bind to the

mutant enzyme or whether it binds but does not displace water from the heme iron (a reason why the spectral response is observed). If the latter is the case, the K_d could be determined by the filter assay. We also used the spectral assay to characterize mutants that had significantly decreased amplitude of the spectral response to gain insight into whether less cholesterol is present inside the enzyme active site as a result of mutation or whether less water is displaced (37). Thus, the filter assay could be used by those who study cholesterol binding proteins as well as by those who work with different P450 enzymes.

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