Use of Cytochrome P-450$_{scc}$ To Measure Cholesterol–Lipid Interactions†

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ABSTRACT: The interaction of cholesterol with phospholipids has been studied with a variety of techniques; however, the possible consequences of such interactions in vivo have not been demonstrated. In this study, the cholesterol-dependent absorbance spectrum of cytochrome P-450$_{scc}$ was used to monitor cholesterol availability in both micellar and vesicular environments. By use of this approach, in conjunction with titration of putative cholesterol binding species, a tight, approximately equimolar complex of cholesterol and digitonin was demonstrated. Sphingomyelin (SM) (both the synthetic *N*-palmitoyl and bovine brain forms) gave sigmoidal titration curves, suggesting a cooperative interaction between this lipid and cholesterol. The interaction of bovine brain glycerolipids and cholesterol was weaker than that of SM and showed no cooperativity. The importance of the phospholipid head group in these interactions was established by the differences in the ability of synthetic 1-palmitoyl-2-oleoylphosphatidylcholine, -phosphatidylethanolamine, and -phosphatidylserine to affect cholesterol availability. Comparison of these results with those of the bovine brain phospholipids indicates that the acyl chain composition of these molecules is also important to these interactions. Titrations of SM in phospholipid vesicles containing cytochrome P-450$_{scc}$ and different types of phosphatidylcholine established that the SM–cholesterol interactions also occur in a bilayer membrane. This study demonstrates that the association of cholesterol with cytochrome P-450$_{scc}$ is inhibited by concentrations of SM commonly found in biological membranes. Therefore, such cholesterol–lipid interactions can potentially affect the function of membrane enzymes.

The appearance of cholesterol in many biological membranes correlates with the presence of sphingomyelin (Patton, 1970), and it has been proposed that these two lipids interact to affect their distribution in vivo. Early model studies by Finean (1953) and Vandenheuvel (1963) suggested a molecular basis for such interactions; nonetheless, direct proof that these exist and have functional significance has been slowly forthcoming. Cholesterol has been shown by Langmuir trough measurements to condense phospholipid monolayers (Demel et al., 1972), perhaps by interacting with surrounding lipids through hydrogen bonds involving its 3β-hydroxyl. In studies employing a variety of techniques, including NMR (Yeagle et al., 1975), ESR (Presti & Chan, 1982), IR, and Raman spectroscopy (Bush et al., 1980), no hydrogen bonding was detected; therefore, the major interacting forces may involve the hydrophobic portion of the lipid molecules. Evidence from differential scanning calorimetry indicates that phospholipid head-group effects cannot be disregarded (van Dijck et al., 1976; Demel et al., 1977; van Dijck, 1979).

Cholesterol–phospholipid interactions have also been studied by measuring the rate of sterol exchange between membranes of different compositions (Bloj & Zilversmit, 1977; Nakagama et al., 1979; Lange et al., 1979; Wattenberg & Silbert, 1983; Fugler et al., 1985). Although somewhat dependent on the source of the membranes and type of phospholipids used, the results of such experiments are consistent with the existence of preferential phospholipid–cholesterol interactions. Several reports (Bloj & Zilversmit, 1977; Nakagama et al., 1979; Fugler et al., 1985) indicate that the interaction may also be sensitive to the degree of saturation of the phospholipid acyl chains, with interactions involving saturated chains being stronger than those with unsaturated chains.

While these results suggest the existence of specific lipid–lipid interactions involving cholesterol, no information concerning their functional significance to membrane enzymes has been reported. In the present study, cytochrome P-450$_{scc}$[1] (side-chain cleavage), an integral membrane protein, is used as a reporter of the cholesterol availability within a membrane or micellar environment. This iron-containing hemoprotein of adrenal mitochondria catalyzes the oxidative side-chain cleavage of cholesterol. Substrate binding by the enzyme results in a bathochromic shift in its absorbance spectrum that can be used to measure the relative cholesterol availability in the presence of putative cholesterol binding compounds. This approach allowed measurement of the binding of cholesterol and digitonin [a saponin which forms a tight, noncovalent complex with β-hydroxysterols (Tschesche & Wulff, 1963)] and of interactions between cholesterol and phospholipids.

Experimental Procedures

Materials. Digitonin, polyethylene sorbitan monooleate (Tween 80), polyoxyethylene 9-lauryl ether, octyl β-D-glucopyranoside, egg PC, bovine brain PE, bovine brain PS, bovine brain PC, bovine brain SM, and *N*-palmitoyl-D-SM were purchased from Sigma; synthetic 1-palmitoyl-2-oleoyl-PS, 1-palmitoyl-2-oleoyl-PE, 1-palmitoyl-2-oleoyl-PC, diphytanoyl-PC, and beef heart CL were from Avanti Polar Lipids, and cholesterol was obtained from Applied Science.

Protein Purification. Adrenodoxin and cytochrome P-450$_{scc}$ were purified from beef heart adrenal cortex mitochondria as previously described (Seybert et al., 1979; Lambeth & Kamin, 1979). The concentration of adrenodoxin was estimated by

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[1] Abbreviations: scc, side-chain cleavage; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; CL, cardiolipin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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using an extinction coefficient of 11 mM^{-1} cm^{-1} at 414 nm. The cytochrome P-450_{scc} concentration was determined from the reduced CO minus reduced difference spectrum using $\Delta\epsilon$ = 91 mM^{-1} cm^{-1} for A_{450} minus A_{490}. Cytochrome P-450_{scc} was stored in small aliquots in buffer (20 mM Hepes, pH 7.2, 100 mM NaCl, and 0.1 mM dithiothreitol) at −80 °C. Under these conditions, the enzyme is stable in both activity and spectral properties for greater than 1 year. Typical preparations contain approximately 1 nmol of cholate and 2–3 nmol of phosphate per nanomole of protein.

Preparation of Mixed Micelles. The appropriate amounts of CL and cholesterol (in chloroform) were mixed in a glass test tube (13 × 100 mm). In some cases, PE, PS, or PC (in chloroform) was included. Solvent was evaporated under a stream of dry nitrogen. The residue was resuspended in a small amount of ether and redried. In experiments requiring either digitonin or SM, the desired quantity (digitonin in methanol, SM in ethanol) was added to the tube and the solvent evaporated under vacuum. Detergent-containing buffer (20 mM Hepes, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% Tween 80, pH 7.0) was then added to the dried lipid mixture to give the appropriate final concentrations of Tween 80 (790 μM), cardiolipin (20 μM), and cholesterol (15 μM). The tube was then sealed under nitrogen and sonicated in a Beuhler Ultramet III bath sonicator until a clear solution was obtained.

Preparation of Phospholipid Vesicles. Various amounts of PC (up to 2 mg/mL depending on the SM concentration), cholesterol (0.2 mol/mol of phospholipid), and CL [30% of the total phospholipid (w/w)] (all in chloroform) were mixed in a glass tube (13 × 100 mm), and the solvent was evaporated under a stream of dry nitrogen. Bovine brain SM (in ethanol) was then added in amounts such that the combined SM and PC concentration was 70% of the total phospholipid amount (w/w). After being dried under vacuum and addition of buffer (20 mM Hepes, 100 mM NaCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol, pH 7.0), vesicles were prepared by sonication as described above for the mixed micelles.

Reconstitution of Cytochrome P-450_{scc} with Phospholipids and Cholesterol. Cytochrome P-450_{scc} was added to detergent micelles or liposomes preequilibrated to the desired temperature to yield a final enzyme concentration of 0.5 μM. Where indicated, adrenodoxin (10 μM) was included in the final micelle–enzyme mixture.

Spectrophotometric Measurements. A Varian 219 spectrophotometer was used to record all optical spectra. The relative amount of cholesterol associated with cytochrome P-450_{scc} was determined by the difference in the Soret maximum of the enzyme at 416 nm (low spin, substrate free) and the 404-nm isosbestic point. Data are expressed as $\Delta A_L - \Delta A_0$, where ΔA_L represents the absorbance difference of the sample containing the cholesterol binding compound (digitonin, SM, PC, PE, or PS) and ΔA_0 is the absorbance difference of the reference (only cholesterol and CL present). Therefore, higher values of $\Delta A_L - \Delta A_0$ reflect a lesser amount of cholesterol bound to the enzyme. In cases where experiments using different enzyme preparations are illustrated in the same figure, the amount of cholesterol associated with the enzyme is expressed as a percentage of the completely low-spin absorbance difference. Therefore, 100% represents no cholesterol associated with the enzyme.

RESULTS

Ability of Cytochrome P-450_{scc} To Measure Relative Cholesterol Availability. Association of cholesterol with cytochrome P-450_{scc} either in membranes or in detergent micelles results in conversion of the heme iron from the low- to the high-spin state. The initial experiments were performed in a simplified mixed micelle system developed by Pember et al. (1983).

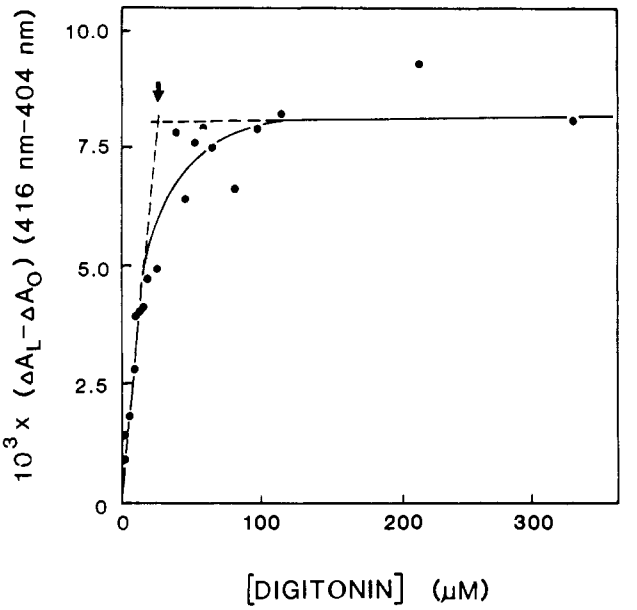

FIGURE 1: Titration of cytochrome P-450_{scc} with digitonin. The difference in absorbance at 416 nm (low spin, substrate free) and 404 nm (isosbestic point) is shown as increasing amounts of digitonin are present in detergent micelles. The arrow indicates the extrapolated breakpoint of the titration at 25 μM digitonin.

When added in increasing amounts of cytochrome P-450_{scc} in micelles containing 15 μM cholesterol, digitonin caused a shift in the optical spectrum from high to low spin (Figure 1). By 100 μM, no substrate was associated with the enzyme (50% inhibition occurred at 14 μM). The extrapolated equivalence point was 25 μM, indicating tight binding of cholesterol by digitonin with an apparent stoichiometry of 1:1.7. Because the concentration of the free vs. micellar digitonin is not known, this represents an upper limit to the binding stoichiometry. Moreover, since commercially available digitonin is only 70–80% pure, the binding ratio is probably nearer to 1:1. This stoichiometry was confirmed by repeating the titration with twice as much cholesterol (30 μM), which shifted the titration curve to the right with an end point at approximately 60 μM (data not shown).

To determine if these interactions are affected by temperature, digitonin titrations were carried out between 4 and 37 °C. A moderate temperature dependence of the cholesterol–enzyme interaction in the absence of any digitonin was observed: however, there were no additional effects when saponin was present (data not shown). Continuous monitoring of the samples at 416 and 404 nm (isosbestic point) revealed that the time required for the spectrum to stabilize at room temperature (22 °C) after addition of enzyme was about 3 min. Therefore, all spectra were collected 4 min after addition of cytochrome P-450_{scc}.

Specificity of the Cholesterol–Digitonin Interaction. Digitonin consists of a steroid-like ring system with five carbohydrate residues extending from the 3-hydroxyl. Because it could be acting simply as a detergent, a titration was performed in which the saponin was replaced by octyl β-D-glucopyranoside, a compound also having a nonpolar region and a sugar head group. No change in cholesterol binding by the enzyme was seen with up to 300 μM octyl β-D-glucopyranoside.

Interaction of Cholesterol with Sphingomyelin. Having demonstrated that cytochrome P-450_{scc} could detect interac-

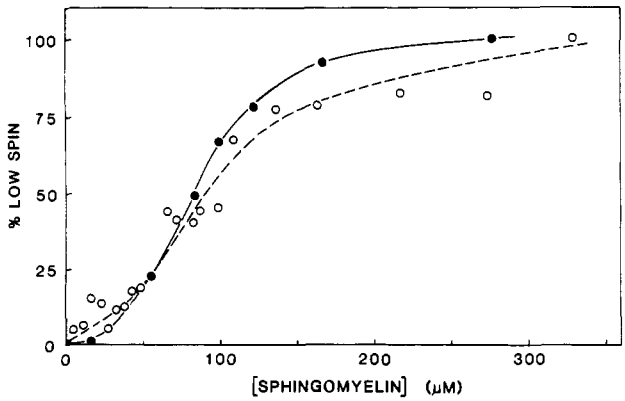

FIGURE 2: Titration of cytochrome P-450$_{scc}$ with sphingomyelin. The relative amount of low-spin heme absorbance (substrate free) is shown as either bovine brain SM (open circles) or *N*-palmitoyl-SM (closed circles) is added to the micelle solution.

tions between cholesterol and other components of mixed micelles, this system was then used to look for interactions with SM. Titrations with bovine brain SM (Figure 2, open circles) and synthetic N-palmitoyl-SM (Figure 2, closed circles) yielded sigmoidal curves with 50% inhibition of cholesterol binding at approximately 85 μM and essentially complete displacement at concentrations greater than 200 μM. Both compounds behaved similarly despite difference in acyl chain composition. The dependence of the SM–cholesterol interaction on the sterol concentration was tested with twice as much cholesterol (30 μM), resulting in a shift in the sigmoidal curve to 50% inhibition at 105 μM and the high-spin spectrum being completely lost by approximately 300 μM SM (data not shown).

Specificity of the Cholesterol–Sphingomyelin Interaction. To test whether these results might be due to the physical characteristics of the Tween 80 mixed micelle rather than specific interactions of the lipid and cholesterol, varying amounts of bovine brain SM were added with a different detergent, polyoxyethylene 9-lauryl ether (0.05%). The resulting curve (not shown) was essentially identical with Figure 2, supporting the conclusion that the observed effects are due to SM.

Since CL enhances the binding of cholesterol by the enzyme (Lambeth, 1981; Pember et al., 1983), inhibition of cholesterol binding by SM might be due to competition for the CL binding site rather than an association with cholesterol. When the titration with bovine brain SM was performed with a 3-fold higher concentration of CL (30 μM), the high-spin spectrum was completely lost at SM concentrations identical with those in Figure 2. Therefore, the phospholipid effects on cholesterol could not be explained by competition for CL binding to cytochrome P-450$_{scc}$.

Another possible explanation for the inhibition by SM is diluted of the cholesterol or its trapping in a separate micelle population not containing the cytochrome P-450$_{scc}$. The former is unlikely because the amount of SM added to achieve complete displacement of the sterol (200 μM) increases the concentration of the hydrophobic components by only 20% (0.1% Tween 80 = 790 μM). To test the latter possibility, adrenodoxin was added to the enzyme-containing micelles. The 1:1 tight binding of this electron transfer protein to cytochrome P-450$_{scc}$ is known to enhance the cholesterol binding affinity of the enzyme by a factor of 20 (Lambeth et al., 1980a), with a corresponding shift toward high-spin cytochrome. Addition of this component effected a significant shift in the absorbance spectrum toward more high-spin character (see Figure 3) when various concentrations of SM were present, indicating cholesterol was still accessible to cytochrome P-450$_{scc}$.

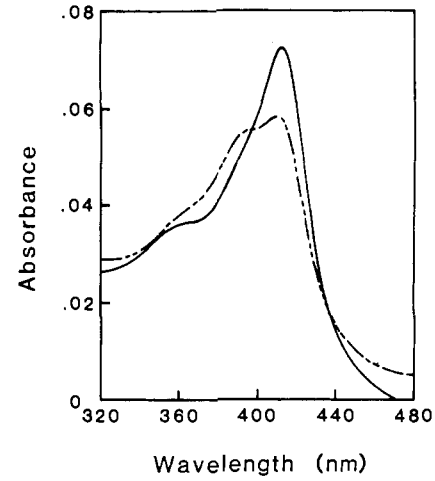

FIGURE 3: Absorbance spectrum of cytochrome P-450$_{scc}$ before and after addition of adrenodoxin. The spectrum of the enzyme in the presence of cholesterol (15 μM) and *N*-palmitoyl-SM (55 μM) is shown by the solid line while the broken line shows the spectrum after the addition of adrenodoxin (10 μM).

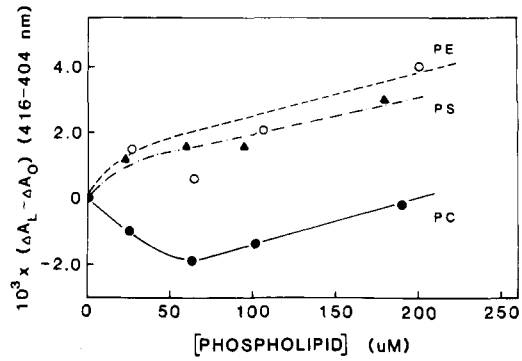

FIGURE 4: Titration curves of cytochrome P-450$_{scc}$ with bovine brain phospholipids. The effect of PC (closed circles), PS (triangles), and PE (open circles) on cholesterol binding by cytochrome P-450$_{scc}$ in the mixed micelle system is shown.

Interactions of Cholesterol with Phospholipids of Different Classes. The mixed micelle system was used to measure the interaction of cholesterol with phospholipids of a different class, the glycerolipids. The effects of bovine brain PC, PE, and PS on the relative availability of cholesterol in the micellar system are shown in Figure 4. Comparison with Figure 2 shows that these lipids apparently interact much less with cholesterol than did bovine brain SM. In addition, the titration curves of the glycerolipids show no sigmoidal character.

Effects of Differences in Head-Group and Acyl Chain Composition on Cholesterol–Lipid Interactions. Because the bovine brain phospholipids include a heterogeneous mixture of acyl chain lengths and degrees of saturation, the specific effects of head-group differences on cholesterol–phospholipid interactions cannot be determined with these lipids. Synthetic preparations of 1-palmitoyl-2-oleoyl-PC, -PE, and -PS were tested. This acyl chain composition was chosen because it is thought to promote the greatest cholesterol–phospholipid interaction (Fugler et al., 1985). As shown in Figure 5, the inclusion of these lipids resulted in 50% inhibition of cholesterol binding by cytochrome P-450$_{scc}$ at 77, 62, and 143 μM, respectively. PC inhibited the cholesterol–enzyme interaction completely at 200 μM while PE and PS were unable to sequester the sterol fully even at the highest concentration tested.

The effect of differences in acyl chain composition on cholesterol–lipid interactions is apparent from comparison of

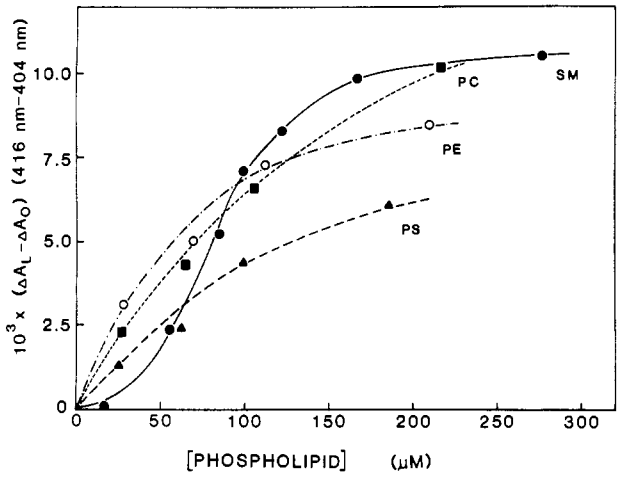

FIGURE 5: Titration curves of cytochrome P-450$_{scc}$ with various phospholipids. The low- and high-spin absorbances were measured upon addition of synthetic *N*-palmitoyl-SM (closed circles), 1-palmitoyl-2-oleoyl-PC (squares), 1-palmitoyl-2-oleoyl-PE (open circles), and 1-palmitoyl-2-oleoyl-PS (triangles) as described (see Experimental Procedures).

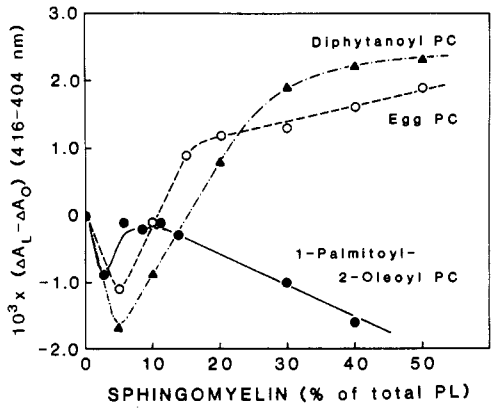

FIGURE 6: Titration of cytochrome P-450$_{scc}$ reconstituted into phospholipid vesicles with sphingomyelin. The change in cholesterol binding by the enzyme is shown as increasing amounts of bovine brain SM are added to phospholipid vesicles consisting of diphytanoyl-PC (triangles), egg PC (open circles), or 1-palmitoyl-2-oleoyl-PC (closed circles).

the results of the titrations of the bovine brain phospholipids (Figure 4) with those of the synthetic phospholipids (Figure 5). The most striking difference is seen with PC. While synthetic PC (200 μM) fully inhibited the interaction of cholesterol with cytochrome P-450$_{scc}$, the bovine brain lipid enhanced the binding of the sterol by the enzyme. The interactions of brain PE and PS with cholesterol are much weaker than those of their corresponding synthetic forms.

Cholesterol–Phospholipid Interactions in Phospholipid Vesicles. To determine whether the interactions observed in the detergent micelles also occurred in bilayer membranes, cytochrome P-450$_{scc}$ was reconstituted into vesicles of varying proportions of bovine brain SM and PC. These experiments are complicated by interactions of both SM and PC with cholesterol; therefore, different species of PC previously shown to have varying effects on cholesterol (Fugler et al., 1985) were examined. Figure 6 shows that binding of cholesterol by the enzyme was inhibited by increasing concentrations of SM when diphytanoyl-PC or egg PC was used. No inhibition was seen with 1-palmitoyl-2-oleoyl-PC, suggesting this lipid has a similar affinity for cholesterol as SM. Although the titration curves in the phospholipid vesicles are more complicated than those seen in the micellar system, they appear to contain a sigmoidal component; therefore, SM is probably affecting cholesterol availability in a similar manner in micelles and bilayer membranes.

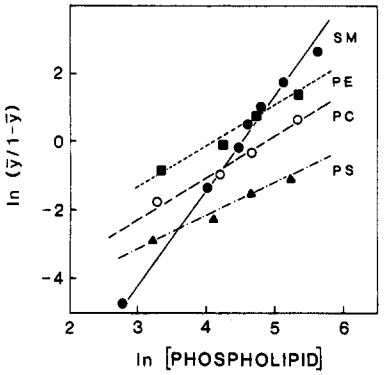

FIGURE 7: Hill plots of titration curves of cytochrome P-450$_{scc}$ with synthetic phospholipids. The Hill coefficients were determined for the titrations of 1-palmitoyl-2-oleoyl-PC (open circles), 1-palmitoyl-2-oleoyl-PE (squares), 1-palmitoyl-2-oleoyl-PS (triangles), and *N*-palmitoyl-SM (closed circles). The quantity $\bar{y}$ represents the percentage of total low spin at that concentration.

DISCUSSION

In the present studies, we have investigated lipid–lipid interactions using cytochrome P-450$_{scc}$ to report the relative availability of cholesterol. Conditions were selected to minimize direct lipid effects on cytochrome P-450$_{scc}$ (Lambeth et al., 1980b; Lambeth, 1981). Although other lipids can interact with the enzyme to perturb the substrate binding equilibrium, CL has been found to be a specific effector of cholesterol binding to the enzyme (Lambeth, 1981; Pember et al., 1983). Therefore, saturating concentrations of CL have been included to fill this effector lipid site. Since the binding results were independent of the CL concentration, this approach was apparently effective at detecting cholesterol–lipid and not enzyme–phospholipid interactions.

The validity of this approach was further demonstrated by using digitonin. This saponin sequestered cholesterol from the enzyme with a stoichiometry near that of the 1:1 complex found by others (Steiner & Holtzem, 1955; Tayaki et al., 1982; Nishikawa et al., 1984). The inability of octyl β-D-glucopyranoside, another neutral glycodetergent, to perturb the cholesterol–enzyme interaction further established that these results were not due to a nonspecific detergent effect on micellar structure.

The mixed micelle system detected clear interactions between cholesterol and various phospholipids, although most were less tight than the digitonin–cholesterol complex. SM showed a sigmoidal concentration dependence, suggesting a cooperative interaction between this phospholipid and the sterol. Hill plots of the titration results (Figures 5 and 7), derived by using extrapolated end points for each titration determined by double-reciprocal analysis, yielded a Hill coefficient for SM of 1.4 while 1-palmitoyl-2-oleoyl-PC, -PE, and -PS had coefficients of 0.6, 0.6, and 0.5, respectively. This indicates that the nature of the SM–cholesterol interaction may differ from that with glycerolipids, perhaps because SM possesses functional groups required for this interaction, such as the 3-hydroxyl or amide-linked fatty acid.

The sigmoidal shape of the SM concentration dependent suggests that this phospholipid must be present in an excess of cholesterol. Yedgar et al. (1974) have suggested that mixed micelles do not form in Triton X-100 with less than 21 mol % SM. Therefore, when SM is present at less than 21 mol %, two populations of micelles (one with no SM and the other

with 21 mol % SM) will exist. Assuming Tween 80 alone forms micelles of 60 monomers (Helenius et al., 1979), with 790 μM Tween 80 and 15 μM cholesterol, there will be an average of about one sterol per micelle. If mixed micelles form at 21 mol % SM (approximately 13 molecules per micelle), very few micelles will contain this lipid at low concentrations of SM. Only cholesterol in these same micelles will be sequestered because cytochrome P-450$_{scc}$ has been shown to interact only with cholesterol in the same membrane (Seybert et al., 1979). At 10 mol % (which was also the concentration where SM inhibited cholesterol binding by 50%, cf. Figure 2), half of the micelles contain SM. While our results are consistent with this behavior, they do not rule out the possibility that SM is evenly distributed among the micelles but must exist in higher order structures (e.g., hexagonal arrays) to sequester cholesterol. Robson and Dennis (1979, 1983) have concluded that mixtures of sphingomyelin and Triton X-100 in varying mole ratios yield mixed micelles of heterogeneous composition, not a mixture of pure surfactant micelles and mixed micelles.

Of the bovine brain phospholipids tested, only SM exhibited significant interaction with cholesterol. The concentration necessary to fully sequester the sterol away from cytochrome P-450$_{scc}$, 200 μM, represents approximately 20% of the micelle composition (the total concentration of detergent, CL, and cholesterol is slightly more than 800 μM). Many biological membranes are known to contain this phospholipid in these or higher proportions (Boggs, 1980). Therefore, the interactions between cholesterol and SM measured by cytochrome P-450$_{scc}$ probably exist in vivo. The interaction of 1-palmitoyl-2-oleoyl-PC with cholesterol was similar to that of SM, which is consistent with results of studies of the rate of cholesterol exchange with vesicles containing *N*-palmitoyl-SM or PC of similar acyl chain length (dipalmitoyl-PC) (Lange et al., 1979).

As has been observed earlier (Bloj & Zilversmit, 1977; Nakagama et al., 1979; Fugler et al., 1985), the interactions with glycerolipids were highly dependent of the acyl chain composition. 1-Palmitoyl-2-oleoyl-PC was capable of fully sequestering cholesterol away from cytochrome P-450$_{scc}$, but the same amount of PC from bovine brain did not alter the sterol–enzyme interaction. That the head group of the phospholipid also plays a role in the interaction with cholesterol is obvious from the titration curves of the 1-palmitoyl-2-oleoylglycerolipids (Figure 5). Whether differences in head groups or acyl chain composition are more important in determining the affinity of a lipid for cholesterol cannot be determined from these results. However, that both these factors are important in these interactions may explain some of the differences in the relative affinities of phospholipids for cholesterol previously reported (Lange et al., 1979; van Dijck, 1979; Fugler et al., 1985).

These interactions were equally apparent when cytochrome P-450$_{scc}$ was reconstituted into phospholipid vesicles (Figure 6). Therefore, cytochrome P-450$_{scc}$ can measure the cholesterol–lipid interactions in both highly curved (detergent micelles) and less curved structures (phospholipid vesicles). Increasing concentrations of SM resulted in inhibition of the interaction between cholesterol and the enzyme when egg PC or diphytanoyl-PC was used. Because there is no SM-induced inhibition of sterol binding in the presence of 1-palmitoyl-2-oleoyl-PC, it appears that this lipid interactions with cholesterol as well or better than SM. Alternatively, it could be that mixtures of SM with egg PC or diphytanoyl-PC undergo phase separation while SM and 1-oleoyl-2-palmitoyl-PC do not. The mixing of these two phospholipids could give rise to an increased interaction between cytochrome P-450$_{scc}$ and cholesterol. The relative affinity of these phospholipids found in the vesicular system (1-palmitoyl-2-oleoyl-PC = bovine brain SM > egg PC > diphytanoyl-PC) agrees exactly with that determined by Fugler et al. (1985) measuring rates of sterol exchange between vesicles.

These findings suggest that, despite the mobility of individual lipids in the membrane bilayer, lipid–lipid interactions can limit their availability to enzymes. In this context, it is interesting to note that the SM content of the inner mitochondrial membrane is very low and should not affect the ability of cytochrome P-450$_{scc}$ to catalyze the side-chain cleavage of cholesterol. However, in other organelles, cholesterol–lipid interactions may have a major impact on membrane enzyme function.

Registry No. 1-Palmitoyl-2-oleoyl-PS, 79980-16-8; 1-palmitoyl-2-oleoyl-PE, 10015-88-0; 1-palmitoyl-2-oleoyl-PC, 6753-55-5; diphytanoyl-PC, 64626-70-6; cytochrome P-450$_{scc}$, 9035-51-2; cholesterol, 57-88-5.

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Human C$\bar{1}$ Inhibitor: Primary Structure, cDNA Cloning, and Chromosomal Localization†

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ABSTRACT: The primary structure of human C$\bar{1}$ inhibitor was determined by peptide and DNA sequencing. The single-chain polypeptide moiety of the intact inhibitor is 478 residues (52 869 Da), accounting for only 51% of the apparent molecular mass of the circulating protein (104 000 Da). The positions of six glucosamine-based and five galactosamine-based oligosaccharides were determined. Another nine threonine residues are probably also glycosylated. Most of the carbohydrate prosthetic groups (probably 17) are located at the amino-terminal end (residues 1–120) of the protein and are particularly concentrated in a region where the tetrapeptide sequence Glx-Pro-Thr-Thr, and variants thereof, is repeated 7 times. No phosphate was detected in C$\bar{1}$ inhibitor. Two disulfide bridges connect cysteine-101 to cysteine-406 and cysteine-108 to cysteine-183. Comparison of the amino acid and cDNA sequences indicates that secretion is mediated by a 22-residue signal peptide and that further proteolytic processing does not occur. C$\bar{1}$ inhibitor is a member of the large serine protease inhibitor (serpin) gene family. The homology concerns residues 120 through the C-terminus. The sequence was compared with those of nine other serpins, and conserved and nonconserved regions correlated with elements in the tertiary structure of α1-antitrypsin. The C$\bar{1}$ inhibitor gene maps to chromosome 11, p11.2-q13. C$\bar{1}$ inhibitor genes of patients from four hereditary angioneurotic edema kindreds do not have obvious deletions or rearrangements in the C$\bar{1}$ inhibitor locus. A *Hgi*AI DNA polymorphism, identified following the observation of sequence variants, will be useful as a linkage marker in studies of mutant C$\bar{1}$ inhibitor genes.

C$\bar{1}$ inhibitor is a highly glycosylated 104 000-Da[1] plasma protease inhibitor that can inhibit components of the complement, coagulation, fibrinolytic, and kinin-releasing systems. It was first purified in 1961 (Pensky et al., 1961) and later (Pensky & Schwick, 1969) found to be immunologically identical with a previously characterized α2-neuraminoglycoprotein (Schultze et al., 1962). C$\bar{1}$ inhibitor has been shown to inhibit macromolecular C$\bar{1}$, the C$\bar{1}$s and C$\bar{1}$r subcomponents of the first component of complement (Ratnoff & Lepow, 1957; Levy & Lepow, 1959; Lepow & Leon, 1962; Gigli et al., 1968; Pensky et al., 1961; Ratnoff et al., 1969), factors XIIa and XIa (Forbes et al., 1970), plasma kallikrein (Ratnoff et al., 1969; Gigli et al., 1970), and plasmin (Ratnoff et al., 1969). Like other serine protease inhibitors [serpins (Carrell, 1984)] of the antithrombin III–α1-antitrypsin family (Petersen et al., 1979), C$\bar{1}$ inhibitor reacts with target proteases to form proteolytically inactive, stoichiometric 1:1 complexes that are stable during $NaDodSO_4$–PAGE (Harpel & Cooper, 1975; Sim et al., 1979; Sim et al., 1980) under reducing conditions (Nilsson et al., 1983). To improve understanding of how C$\bar{1}$ inhibitor regulates diverse plasma serine proteases, we have determined its sequence and covalent structure. To address questions concerning the evolution and structure of

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[1] Abbreviations: bp, base pairs; kb, kilobase; Da, dalton; serpin, serine protease inhibitor; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HANE, hereditary angioneurotic edema; Å, angstrom; DNS, 5-(dimethylamino)naphthalenesulfonyl; RFLP, restriction fragment length polymorphism; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

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