Effects of Cholesterol on the Function and Thermotropic Properties of Pure UDP-glucuronosyltransferase*

Michal Rotenberg$ and David Zakim$  

From the Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, New York 10021

The effects of cholesterol on the activity and thermal properties of a pure, delipidated isofrom of UDP-glucuronosyltransferase were examined after incorporation of enzyme into unilamellar bilayers of distearoylphosphatidylcholine (DSPC) or dioleoylphosphatidylcholine (DOPC). Cholesterol, in bilayers of DSPC, decreased enzyme activity and lowered the temperature (from 37 to 30 °C) for a reversible transition from the active form of the enzyme to a less active form. These effects could be separated from each other in that the effect on reversible inactivation of the enzyme occurred at lower concentrations of cholesterol than the effect on activity of the active form of the enzyme. In addition, cholesterol in bilayers of DSPC stabilized UDP-glucuronosyltransferase against irreversible thermal inactivation. The extent of stabilization increased with increasing concentration of cholesterol in the bilayers. The effects of cholesterol on UDP-glucuronosyltransferase depended, however, on the nature of the bilayer containing cholesterol. Cholesterol had small effects, if any, on the properties of UDP-glucuronosyltransferase in bilayers of DOPC.

UDP-glucuronosyltransferases (EC 2.4.1.108) are integral components of membranes, being most abundant in the microsomal fraction of liver (Dutton, 1980). For at least two isofroms purified from pig liver (Hochman and Zakim, 1983; Magdalou et al., 1982), several functions are modulated by the composition of the lipid matrix in which pure enzyme is inserted, including $k_{	ext{cat}}$ (Magdalou et al., 1982), avidity for binding of substrates (Hochman et al., 1981), allosteric activation (Hochman and Zakim, 1983), and thermal stability (Rotenberg and Zakim, 1989). The lipid-dependent regulation of pure enzyme may be important for understanding regulation of UDP-glucuronosyltransferase within its natural membrane location because changes in function induced by varying the lipid environment of pure enzyme have counterparts for enzyme in microsomes. For example, activity, avidity for substrates, response to allosteric regulators, and thermal stability can be altered by addition of a variety of detergents to microsomes (Hochman and Zakim, 1983; Vessey and Zakim, 1971; Zakim et al., 1973; Zakim and Vessey, 1975), suggesting that the functional state of UDP-glucuronosyltransferase in situ is modulated by lipid–enzyme interactions (Dannenberg et al., 1990a, 1990b; Vessey and Zakim, 1971; Zakim and Vessey, 1975). Understanding the regulation of UDP-glucuronosyltransferase in model systems and eventually in the endoplasmic reticulum of a living animal is likely to depend, therefore, on determining how different lipids interact with the enzyme and influence its catalytic function.

Cholesterol is a significant component of total liver microsomal lipid, the molar ratio of cholesterol/phospholipid being 0.12 (Bruckdorfer and Graham, 1976). Although cholesteryl phosphocholine, which forms micelles, activates delipidated UDP-glucuronosyltransferase (Zakim et al., 1988), this system probably has limited application to understanding the effects of cholesterol on enzyme embedded in a bilayer, which is the physiological matrix of the enzyme. When present in the environment of other integral membrane enzymes, cholesterol usually inhibits activity, except perhaps for membrane proteins with a transport function (Brasitus et al., 1988; Cheng et al., 1986; Saito and Silbert, 1979; Warren et al., 1975). Hence, interactions with cholesterol might account for the observation that the activity of UDP-glucuronosyltransferase in untreated microsomes is constrained as compared with activity in modified microsomes (Leuders and Kuff, 1967; Zakim and Vessey, 1971). On the other hand, Castuma and Brenner (1986a) have reported that addition of cholesterol to microsomes from guinea pig liver increased the activity of UDP-glucuronosyltransferase at V_{	ext{max}} and that supplementation of diet with cholesterol, which increased the cholesterol content of guinea pig liver microsomes, led to similar modifications of the enzyme (Castuma and Brenner, 1986b). In the present work, we report the effects of cholesterol on the function of UDP-glucuronosyltransferase, using a pure isoform designated GT_{ls} (Magdalou et al., 1982), that was reconstituted into unilamellar lipid vesicles (ULVs). These data are important because they demonstrate the effects of cholesterol on the properties of UDP-glucuronosyltransferase in a relatively simple, reconstituted system.

MATERIALS AND METHODS

Phosphatidylcholines were purchased from Avanti Polar Lipids. Cholesterol was purchased from Boehringer Mannheim. All other reagents were analytical grade. The GTls isofrom of UDP-glucuronosyltransferase, purified from pig liver microsomes, was used in all experiments (Hochman et al., 1981; Magdalou et al., 1982). Small ULVs were prepared by sonication (Barenholz et al., 1977). GTls was reconstituted into ULVs as in Scotto and Zakim (1985, 1988). Briefly, this method consists of the spontaneous insertion of pure enzyme into preformed lipid vesicles, with or without cholesterol. In the current experiments, protein was mixed with a 10-fold excess (w/t) of phospholipid as in Scotto and Zakim (1988). Density gradient centrifugation of lipid and protein mixtures 30 min later (Scotto and

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† Present address: Dept. of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel.

‡ To whom correspondence should be addressed.
Zakim, 1985), to resolve fractions of lipid, lipid-protein complexes, and protein, demonstrated that GTp was completely incorporated into lipid vesicles. About 70% of total lipids also were present as lipid-protein complexes. This yielded lipid-protein complexes with ratios (mole of enzyme/mole of phospholipid) of about 500/1 (lipid/enzyme).

Studies of UDP-glucuronosyltransferase in vesicles with lipid/protein in the range of 30/1 to >200/1 (mole/mole) have shown no dependence of activity on the concentration of UDP-glucuronosyltransferase in vesicles.2

Enzyme activities were assayed at different temperatures, using p-nitrophenol as aglycone, by measuring absorbance changes at 400 nm. The assay mixtures contained 0.05 mM p-nitrophenol and several concentrations of UDP-glucuronic acid, at each of the indicated temperatures, to estimate activity at $V_{max,app}$, as under "Materials and Methods." The data are plotted in the form of the Arrhenius equation.2

RESULT

Effect of Cholesterol on the Activity and Thermotropic Properties of UDP-glucuronosyltransferase in ULVs of DSPC or DOPC—Delipidated GTp undergoes a rapidly reversible and abrupt temperature-dependent change in state from active to inactive forms of the enzyme at about 35 °C (Hochman et al., 1981; Rotenberg and Zakim, 1989). The temperature for the abrupt onset of this transition depends on the acyl chains of the lipid matrix in which UDP-glucuronosyltransferase is embedded (Rotenberg and Zakim, 1989). To separate possible changes in activity secondary to effects on this temperature-dependent change in state from effects due to modulation of the kinetic parameters of the active form of UDP-glucuronosyltransferase, the effects of a given lipid on the catalytic function of GTp must be measured across a range of temperatures. Rates of glucuronidation hence were measured at different temperatures between 23 and 48 °C.

Shown in Fig. 1 are the effects of cholesterol on activities at $V_{max,app}$ (activity extrapolated to infinite concentrations of UDP-glucuronic acid at 0.05 mM p-nitrophenol) for enzyme reconstituted into ULVs of DSPC. The data are plotted in the form of the Arrhenius equation. Data in closed circles are for enzyme in pure DSPC and show an abrupt transition (at 37 °C) from active to inactive enzyme (Rotenberg and Zakim, 1989), which was reversible (data not shown). Although 10 mol % cholesterol (open circles in Fig. 1) had no effect on the activity of GTp at temperatures below 35 °C, this concentration of cholesterol lowered the temperature for the onset of the transition between active and inactive forms of GTp. As for enzyme in pure DSPC, the temperature-dependent decline in activity was reversible for enzyme in ULVs containing cholesterol. In addition, the transition between states, which can be described usually by a simple two-state system, e.g. $E \leftrightarrow E'$, for enzyme in pure DSPC (Rotenberg and Zakim, 1989), appeared to be more complex for enzyme in DSPC plus 10 mol % cholesterol. Instead of a smooth decline of activity as a function of temperature for GTp, in pure DSPC, there was no variation of activity with temperature between 37 and 41 °C, and the decline of activity versus temperature for enzyme in DSPC plus 10 mol % cholesterol appeared to be discontinuous at 37 and at 41 °C.

The activity of GTp at $T < 30$ °C was unaffected in ULVs of DSPC with 20 mol % cholesterol, but the temperature for the onset of the transition to a less active form, as reflected by the discontinuity of the Arrhenius plot, decreased to 30 °C. There was almost no effect of temperature on activity between 30 and 37 °C in this system, but a second discontinuity, followed by a steep temperature-dependent decline in activity, occurred at 37 °C.

In contrast to lower concentrations, the activity of GTp at all temperatures was decreased by 30 mol % cholesterol. The temperature of onset for the transition of GTp to a modified less active form of the enzyme was the same for ULVs with 30 or 20 mol % cholesterol; but the flat region of the Arrhenius plot extended over a greater interval of temperature for DSPC plus 30 mol % cholesterol as compared with lower amounts. In addition, the slope for the temperature-dependent decline in activity at $T > 41$ °C was shallower at 30 mol % cholesterol than for lower concentrations. We do not yet understand the significance of this last observation; but a reasonable interpretation of the regions of flatness in the Arrhenius plots for DSPC with > 20 mol % cholesterol is a balance of effects of temperature on the activities of the native and temperature-modified forms of GTp, and on the equilibria for transitions between them. Possibly, however, cholesterol stabilized forms of GTp that do not exist in vesicles without cholesterol.

Comparison of the data in Figs. 1 and 2 shows that the effects of cholesterol on the activity and thermotropic properties of UDP-glucuronosyltransferase depended on the phospholipid into which the protein and cholesterol were incorporated. Thirty mol % cholesterol in ULVs of DOPC had essentially no effect on the properties of GTp. There were

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only small differences in activity at 37 °C for enzyme in pure DOPC or DOPC plus cholesterol. It was not possible to be certain that cholesterol altered the temperature for the onset of the transition $E \leftrightarrow E'$ in ULVs of DOPC.

Effect of Cholesterol on the Stability of Reconstituted UDP-glucuronosyltransferase—As mentioned already, the decrease in activity of UDP-glucuronosyltransferase at temperatures higher than 37 °C reflects a reversible conformational change in the protein (Rotenberg and Zakim, 1989). This inactive form of UDP-glucuronosyltransferase can in turn undergo an irreversible change to an inactive conformer. The effect of cholesterol on the transition of GT$_{2p}$ to an irreversibly denatured enzyme was studied by heating enzyme at 44 °C for relatively long times, and then measuring activity at 37 °C. This was done for enzyme reconstituted into ULVs of DSPC with 0, 20, or 30 mol % cholesterol. The apparent decay of $E$ to irreversibly inactivated enzyme was a first order process, and the rate was a function of the cholesterol content in the membrane. The rate constant for inactivation ($k$) for pure DSPC vesicles was $3.9 \times 10^{-2}$ min$^{-1}$; $k$ values for ULVs of DSPC with 20 and 30 mol % cholesterol were $1.4 \times 10^{-2}$ and $7.7 \times 10^{-3}$, respectively. UDP-glucuronosyltransferase activity in DOPC vesicles at 44 °C was quite stable ($k = 1.2 \times 10^{-5}$). Addition of cholesterol to ULVs of DOPC did not change the rate constant for irreversible inactivation.

**DISCUSSION**

The data presented above show that cholesterol has complex effects on the functional state of GT$_{2p}$. Depending on the concentration of cholesterol in a bilayer, the phospholipids comprising the bilayer, and the temperature of the system, cholesterol can be shown to inhibit the catalytic activity of GT$_{2p}$ to lower the stability of the active form of the enzyme. These two effects can be separated from each other in that the stability of $E$ relative to $E'$ in the temperature-dependent transition $E \leftrightarrow E'$ is modulated by concentrations of cholesterol that do not decrease activity of the $E$ form. Thus, the temperature for the onset of the transition $E \leftrightarrow E'$ was the same for enzyme in DSPC bilayers containing 20 and 30 mol % cholesterol, but only the latter concentration of cholesterol inhibited the activity of GT$_{2p}$ in state $E$. In addition to modulating the activity of the $E$ state of GT$_{2p}$, cholesterol stabilizes the $E'$ form of the enzyme against irreversible thermal denaturation. The effects of cholesterol on GT$_{2p}$ depended, however, on the composition of bilayers. Cholesterol had no demonstrable effects on the properties of GT$_{2p}$ in bilayers of DOPC.

Cholesterol, in theory, could modulate the function of GT$_{2p}$ via direct microscopic interactions with the enzyme, i.e. by inserting into the lipid annulus of the enzyme or via cholesterol-dependent changes in bulk phase viscosity. These two mechanisms may not be mutually exclusive. But, in the absence of direct measurements of the composition of the lipid annulus of GT$_{2p}$ in different lipids, we cannot be certain which of these mechanisms applies. Nevertheless, there are some data to suggest that the observed effects of cholesterol are not due simply to the effects of this lipid on the bulk phase properties of bilayers. For example, to the extent that the issue has been studied, there is an inverse correlation between the activity of GT$_{2p}$ and viscosity of lipid bilayers (Hochman and Zakim, 1983; Rotenberg and Zakim, 1989); and since cholesterol decreases the viscosity of DSPC but increases the viscosity of DOPC bilayers (Hysslop et al., 1990; Stockton and Smith, 1976; Yenagle, 1988), we might have expected cholesterol to increase the activity of GT$_{2p}$ in bilayers of DSPC but decrease activity in bilayers of DOPC, which was not observed.

Stabilization against thermal denaturation has been observed for band 3 protein from red cells with increasing viscosity of bilayers in reconstituted systems (Maneri and Low, 1988). The stabilization of GT$_{2p}$ by cholesterol cannot be explained in this way, however. For, as for changes in activity, the effects of cholesterol on the stability of GT$_{2p}$ do not correlate with the effects of cholesterol on the bulk phase properties of bilayers of DSPC and DOPC. Moreover, the transition $E \leftrightarrow E'$, which is lipid-dependent, is not enthalpy-driven (Rotenberg and Zakim, 1989) and does not correlate with the bulk phase properties of the lipid environment. We believe these observations imply that cholesterol modulated the functional state of GT$_{2p}$ because of direct interactions with it. There are insufficient data at this point, however, for considering why the effects of cholesterol depended on the lipid composition of bilayers.

As mentioned above, Castuma and Brenner (1986a, 1986b) have reported that increasing the concentration of cholesterol in microsomes in _vivo_ or in endoplasmic reticulum in _vivo_ activates UDP-glucuronosyltransferase. In view of the data for the direct effects of cholesterol on the properties of pure enzyme, we think the effects of cholesterol on the activity of UDP-glucuronosyltransferase in microsomes (Castuma and Brenner, 1986a, 1986b) are likely to reflect indirect effects, by changes in the composition of phospholipids in the environment of the enzyme or by another as yet undefined but indirect mechanism.

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