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THERMODYNAMIC PROPERTIES OF THE CHOLESTEROL TRANSFER REACTION FROM LIPOSOMES TO CYTOCHROME $P450_{scc}$: AN ENTHALPY-ENTROPY COMPENSATION EFFECT

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We have investigated the thermodynamic properties of the cholesterol transfer reaction from various types of liposomes to purified steroid-free cytochrome $P450_{scc}$. From the results of temperature effect on the reaction, we obtained $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ values for the reaction with various phosphatidylcholine vesicles containing different fatty acyl chains. The plots of $\Delta H^{\ddagger}$ against $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$ against $\Delta H^{\ddagger}$ both reveal that an enthalpy-entropy compensation effect was seen in a series of phospholipid and micellar media of cholesterol. Our β-value of 420° K is unusually high among reported values for biochemical reactions.

In adrenocortical mitochondria, $P450_{scc}$[1] is the terminal mixed-function oxidase for the cholesterol side chain cleavage reaction, which is known to be stimulated in vivo by the action of ACTH (1). The current hypothesis on the mechanism of action of ACTH suggests that ACTH stimulates the intramitochondrial movement of cholesterol to the cytochrome through the processes involving c-AMP, protein kinase, cholesterol esterase, and ribosomal protein synthesis. In recent years many studies were directed to understand the cholesterol binding reaction to the heme protein (2-5).

In order to gain some insight into the mechanism of the cholesterol binding reaction, we decided to investigate the thermodynamic properties of this reaction by utilizing liposomal cholesterol and the purified cytochrome.

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[1] The abbreviations used are as follows: ACTH, adrenocorticotropic hormone; c-AMP, cyclic AMP; $P450_{scc}$, cytochrome P450 which is specific to the cholesterol side chain cleavage reaction; DLPC, dilauroylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPoPC, dipalmitoleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonoylphosphatidylcholine; and PD, 1,2-propanediol.

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From these studies, we found an enthalpy-entropy compensation effect among a series of reactions with different phospholipid and micellar media of cholesterol.

METHODS AND MATERIALS

$P450_{scc}$ was purified from bovine adrenal cortex according to the method described previously (6). The heme content was 7-8 nmol per mg protein, indicating a single band upon sodium dodecylsulfate gel electrophoresis. Steroid-free $P450_{scc}$ was prepared by the enzymatic method as described elsewhere (7). The heme content of steroid-free $P450_{scc}$ was 7 nmol per mg protein. The yield was about 60% after the removal of steroids. The resulting sample showed a typical low spin-type spectrum of heme protein. Liposomes were prepared by the method of Huang (8). Sonication was performed for 30 min with a Branson Sonifier at temperature above the melting point of phospholipid. Cholesterol (9), phosphorus in phospholipid (10), and heme in $P450_{scc}$ samples (11) were determined by reported methods. Phospholipids were obtained from Avanti, and other chemicals from commercial sources.

RESULTS

Figure 1 shows the spectral changes of steroid-free $P450_{scc}$ upon the binding of cholesterol in DOPC vesicles. These changes are results of the heme iron spin conversion from low to high spin state upon substrate binding (Reaction 1). By the addition of cholesterol in a solution of propanediol, similar changes were observed, except that cholesterol in DOPC vesicles reacted faster than that in propanediol when the initial rates were compared.

$$P450_{scc}\ (\text{low spin}) + \text{cholesterol} \rightarrow P450_{scc}\ (\text{high spin}) - \text{cholesterol} \quad (1)$$

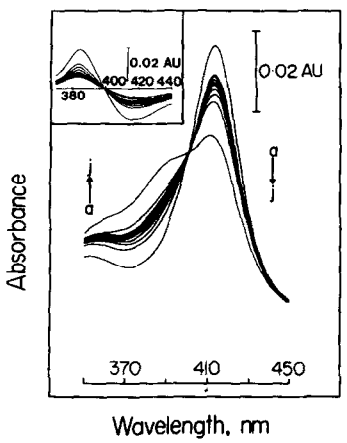

Figure 1. Binding of liposomal cholesterol to steroid-free $P450_{scc}$ DOPC liposomes containing 30 mol % cholesterol were added to 0.45 μM steroid-free $P450_{scc}$ in the reaction mixture of 10 mM K-phosphate buffer, pH 7.4, at 20° C. The molar ratio of cholesterol to $P450_{scc}$ was 3. Spectra (a)-(j) were taken at 0, 0.5, 2, 4, 7, 11, 16, 22, 30, and 40 min after the addition of cholesterol, respectively. The insert shows the difference spectra against zero time spectrum. AU:absorbance unit.

It is important to note the fact that our spectral changes induced by liposomal cholesterol give symmetrical difference spectra (inset of Figure 1). This indicates no denaturation of the cytochrome during the reaction period. In some works, asymmetrical difference spectra were reported, raising a doubt of nativity of the heme protein during the transfer reaction.

When the number of cholesterol-containing vesicles were increased at the constant concentration of cholesterol in the reaction mixture, the binding rate correspondingly increased with a Michaelis-Menten kinetics, suggesting a random collision mechanism or an aqueous medium-mediated mechanism between the cytochrome and vesicles. Above approximately 8 μM liposomal phosphorus concentration, the rate decreased, suggesting mechanisms more complex than a random collision. The following experiments were thus carried out below 8 μM phosphorus concentration.

In order to see the effect of fatty acyl chains in phospholipid on the cholesterol binding reaction, we selected dioleoyl(18:1)$_2$, dipalmitoleoyl (16:1)$_2$, dipalmitoyl (16:0)$_2$, and palmitoylarachidoyl (16:0)(20:4) phosphatidylcholine as phospholipids for liposomal samples. Figure 2 represents the Arrhenius plots of the cholesterol binding reactions by using 46 mol % cholesterol-containing liposomes and steroid-free P450$_{scc}$. From these results, we calculated $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ at 25° C, pH 7.4 for respective reactions (Table I).

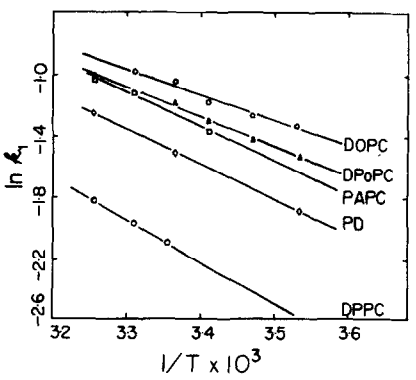

Figure 2. Arrhenius plots of cholesterol binding reactions to steroid-free P450$_{scc}$ in various phospholipid vesicles. Various types of 46 mol % cholesterol-containing liposomes were added to 0.5 μM steroid-free P450$_{scc}$ in 10 mM K-phosphate buffer, pH 7.4, at 20° C. The initial phase of reaction was approximated to a first order rate equation. The molar ratio of cholesterol to P450$_{scc}$ was 3. PD:cholesterol dissolved in propanediol (5 μM as a final concentration).

TABLE I. List of $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ of Cholesterol Binding Reactions to Steroid-Free $P450_{scc}$

Liposome	$\Delta G^{\ddagger}$, kcal/mol	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, eu
DOPC	20.40 ± 0.04	2.8 ± 0.3	-59.1 ± 1.0
DPoPC	20.48 ± 0.03	3.3 ± 0.2	-57.7 ± 0.7
PAPC	20.50 ± 0.04	3.8 ± 0.4	-56.0 ± 1.3
PD	20.65 ± 0.03	4.0 ± 0.1	-55.9 ± 1.0
DPPC	21.01 ± 0.01	4.9 ± 0.01	-54.0 ± 0.1

The cholesterol contents in liposomes were 46 mol %, and the solution in PD was 5 μM as a final concentration.

When these $\Delta H^{\ddagger}$ values were plotted against respective $\Delta S^{\ddagger}$ values, a straight line was obtained with a correlation coefficient of 0.990 and a slope of 403° K (Figure 3).

Krug et al. (12,13) described that $\Delta H^{\ddagger}$ vs $\Delta S^{\ddagger}$ plots often lead to an erroneous conclusion due to propagation of experimental errors when $\Delta S^{\ddagger}$ is

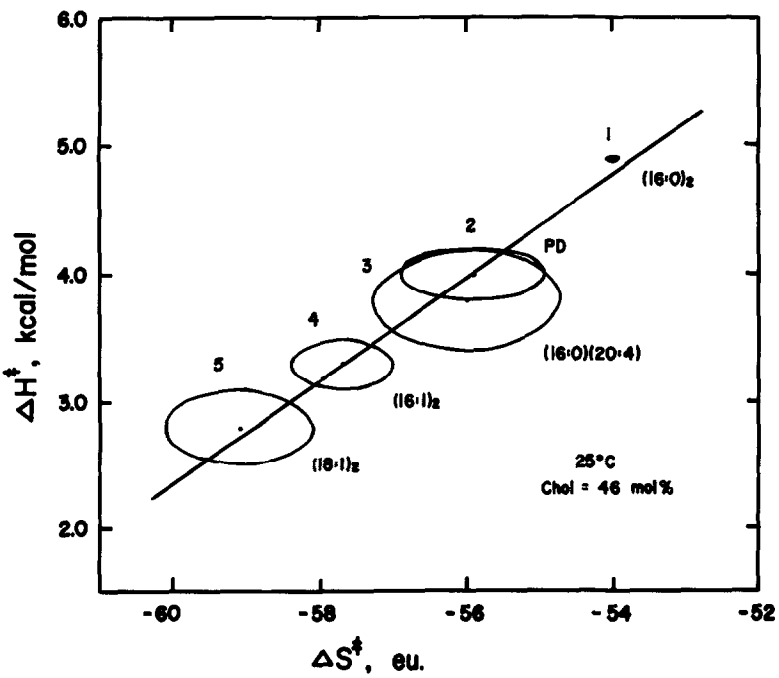

Figure 3. The plot of $\Delta H^{\ddagger}$ against $\Delta S^{\ddagger}$ for the cholesterol transfer reaction from various phosphatidylcholine vesicles to steroid-free $P450_{scc}$. The data were taken from Figure 2. PD:propanediol-dissolved cholesterol (5 μM as a final concentration). The area indicated by circle means the statistical deviations in both parameters. The central point represents the mean value.

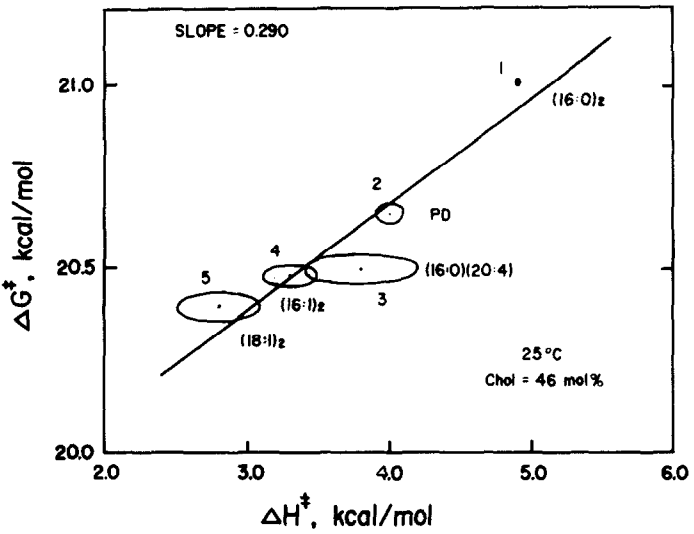

Figure 4. The plot of $\Delta G^{\ddagger}$ against $\Delta H^{\ddagger}$ for the cholesterol transfer reaction from various phosphatidylcholine vesicles to steroid-free $P450_{SCC}$. The data were taken from Figure 2. PD:cholesterol-dissolved in propanediol (5 μM as a final concentration). The area indicated by circle means the statistical deviations in both parameters. The central point represents the mean value.

calculated from Arrhenius plots. They demonstrated on the basis of statistics that the source of errors arises from the use of narrow experimental temperature range (1/T). Therefore, the slope ($\Delta H^{\ddagger}$) is more accurate than the intercept ($\Delta S^{\ddagger}$), and consequently the plots of $\Delta G^{\ddagger}$ against $\Delta H^{\ddagger}$ would provide a safe conclusion. Figure 4 indicates the $\Delta G^{\ddagger}$ vs $\Delta H^{\ddagger}$ plot, which gives a straight line with a correlation coefficient of 0.945. The β-value was calculated to be 420° K from these data (slope = $\frac{\beta - T}{\beta}$). By definition, the reaction is isoentropic when the β-value is ∞, and the reaction is isoenthalpic when the β-value is 0. If the β-value is > 0, the enthalpy-entropy compensation is in effect. Therefore our non-zero β-value is suggestive of this effect, and is probably caused by both conformational changes of membrane and protein and a direct participation of water in the process (14).

DISCUSSION

The reaction of $P450_{SCC}$ with liposomal cholesterol involves at least three different interactions, which are (i) the interaction between the heme protein and cholesterol, (ii) the interaction between cholesterol and phospholipid, and (iii) the interaction between the cytochrome and phospholipid. The

dissociation constant of the complex of $P450_{scc}$ and cholesterol has been estimated to be the order of magnitude of 10^{-6} M (2). Our present knowledge on the affinity of cholesterol to different phospholipids is scant. In short, however, the affinity decreases upon the increase in double bonds in fatty acyl chains. The binding profile of cholesterol to $P450_{scc}$ roughly correlates with the reciprocal of the affinity of cholesterol to phospholipids with some exceptions (2,4). The interaction between the heme protein and phospholipid has been studied previously (2). Phospholipids serve as low spin inducers for the cholesterol-bound cytochrome with spectroscopic dissociation constants on the order of 10^{-3} M (2). The annular phospholipids of the heme protein are known to be phosphatidylcholine and phosphatidylethanolamine with a minor component of cardiolipin (15,16), and this composition is similar to that of bulk phospholipids in adrenocortical mitochondria.

The cholesterol transfer reaction occurs under circumstances involving the above-mentioned three interactions. The values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Table I) perhaps reflect hydrophobic forces among $P450_{scc}$, cholesterol, and phospholipid as sources of the interactions. In addition, changes in the order of solvent water molecules which hydrate both membranes and P450 molecules, van der Waals forces, and hydrogen bonding may be also responsible for our negative value of $\Delta S^{\ddagger}$ (17). The enthalpy-entropy compensation effect in our system includes the reaction mixture with cholesterol in a propanediol solution, where cholesterol molecules disperse as micelles. This means that the $\Delta H^{\ddagger}$-$\Delta S^{\ddagger}$ compensation effect is seen not only in phospholipid environment but also in aqueous medium. As a consequence, the $\Delta G^{\ddagger}$ value is kept relatively constant.

In the literature, the β-values are described for some biological reactions (Table II). Our value of 420^{o} K is significantly higher than values reported for several biochemical reactions, which are carefully selected for kinetic measurements. Our high value may be explained by the fact that the reactions studied by us occur in the environment of the liposomal membrane. Although the detail of the molecular mechanism remains to be elucidated in the future studies, an enthalpy-entropy compensation is seen in a series of

TABLE II. List of $\Delta H^{\ddagger}$-$\Delta S^{\ddagger}$ Compensation Reactions in Biological Systems

Enzymes	Reaction	β(^{o}K)[a]	Reference
$P450_{scc}$	Cholesterol binding	420[b]	this work
Mammalian erythrocytes	Malonamide-induced hemolysis	304-320[c] 308-318[b],[d]	18
Horse metmyoglobin, human and tubifex methemoglobin	Ligand binding	about 319[c],[d]	19
Hemoglobin	Thermal denaturation	265[b],[d]	20,21
α-Chymotrypsin	Acylation by anilides	402[b],[d]	22
Lysozyme	Substrate binding	301[b]	23
α-Chymotrypsin	Substrate hydrolysis	280-300[c]	24,25
Acetylcholinesterase	Ligand binding	288[c]	26

a) For most enthalpy-entropy compensation processes, the β-values lie in a relatively narrow range between 250 to 315o K (14). In the acylation reaction of chymotrypsin by various anilides, the β-value was approximately 400o K. The high value may be due to the involvement of electron rearrangement in the acylation reaction. At present, our value of 420o K is difficult to be explained by a mechanistic viewpoint. However, the contribution of membrane-bound or micellar cholesterol in the high value is likely.
b) from the slope of $\Delta G^{\ddagger}$-$\Delta H^{\ddagger}$ plot.
c) from the slope of $\Delta H^{\ddagger}$-$\Delta S^{\ddagger}$ plot.
d) by our calculation.

cholesterol forms including lipid and aqueous media, suggesting that all reactions applied in this study are basically governed by the same mechanism regardless of fluidity of membrane and nature of medium.

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