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SOLUBILIZATION AND PROPERTIES OF A PHOSPHATIDYLETHANOLAMINE-DEPENDENT METHYLTRANSFERASE SYSTEM FOR PHOSPHATIDYLCHOLINE SYNTHESIS FROM MOUSE LIVER MICROSOMES

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SUMMARY: A method was developed for solubilization of a phosphatidylethanolamine-dependent methyltransferase system for phosphatidylcholine synthesis in mouse liver microsomes. A preparation with high specific activity was obtained in good yield by treating 0.3% deoxycholate-treated microsomes with 0.2% Triton X-100. With this preparation, methyl incorporation into phospholipids was activated by phosphatidylethanolamine and its methylated intermediates. The specific activity of the preparation with phosphatidylethanolamine was 4 times that of intact microsomes. The reaction products with the solubilized preparation were phosphatidylcholine and methylated intermediates of phosphatidylethanolamine.

PC is the main phospholipid of most mammalian cells and it is mainly synthesized by N-methylation of PE with SAM as well as by the CDP-choline pathway (1). The stepwise methylation of PE was first observed in microsomes of rat liver and other organs (2,3). In bacteria this reaction is catalyzed by two membrane-bound enzymes: the "first" enzyme catalyzes addition of the first methyl group and the "second" enzyme catalyzes the other two methylations (4). Recently, the participations of two enzymes in the reaction in mammalian membranes were also demonstrated by Hirata et al. (5,6). In addition a soluble enzyme catalyzing the "first" reaction was also found in bacteria and was shown to have different properties from the microsomal enzymes of mammalian cells (7). We are interested in this methylation enzyme system, since it could cause biological transformations of membranes by altering the polar head group of phospholipid and thus regulating membrane functions, as proposed by Hirata and Axelrod (8).

The following abbreviations are used: PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PME, phosphatidylmonomethylethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; CL, cardiolipin; SAM, S-adenosyl-L-methionine; DOC, sodium deoxycholate; SDS, sodium dodecylsulfate.

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Rehbinder and Greenberg (9) succeeded in solubilizing the "second" enzyme, but not the "first", from rat liver microsomes. Hirata et al. (5) suggested that it was possible to solubilize the "first" enzyme with detergents. This paper reports the solubilization and some properties of the PE-dependent methyltransferase system from mouse liver microsomes.

MATERIALS AND METHODS

Liver microsomes from male ddY mice of 5 weeks old were prepared by a slight modification of the method of Schneider (10). PE and PC were purified from egg yolk (11). PME and PDME were synthesized with carrot plastid phospholipase D (12), or extracted from LM cells (13). PS (bovine brain), CL (bovine heart) and SAM were from Sigma Chemical Co. Phospholipids were dispersed in water by sonication (14). [Methyl-^{14}C]SAM and Triton X-100 were from The Radiochemical Centre, Amersham and Rohm and Haas, respectively. DOC was purchased from Sigma Chemical Co. and was recrystalized (14). Protein (15) and phosphate groups of phospholipids (16) were measured by standard procedures.

Assay of the phosphatide methyltransferase system. The methyltransferase system was assayed by measuring the incorporation of methyl groups from [methyl-^{14}C]SAM into phospholipids. The assay mixture contained 24 μM [methyl-^{14}C]SAM (50 nCi), 10 mM $MgCl_2$, 10 mM L-cysteine, 10% glycerol, 0.1 M Tris-HCl buffer (pH 8.8) and enzyme solution in a total volume of 0.2 ml. The reaction was started by adding radioactive SAM. The mixture was incubated at 37 °C for 30 min and then the reaction was stopped with 3 ml of chloroform/methanol (2/1, v/v). The chloroform layer was washed twice with 0.85% NaCl (2 ml) and then its radioactivity was counted in a liquid scintillation spectrometer. The activity of the boiled enzyme preparation was also assayed and its radioactivity, usually less than 4% of that with the unboiled enzyme preparation, was substracted from that of the latter. The PE-dependent activity was assayed with 0.5 mM PE in the assay mixture.

Analysis of the reaction products. The reaction products were separated by two-dimensional thin-layer chromatography (Silica gel 60, 20 x 20 cm, 0.25 mm thickness, E. Merck) with chloroform/methanol/water (65/25/4, by vol.) as solvent in the first dimension and n-butyl alcohol/acetic acid/water (6/2/2, by vol.) in the second. Autoradiography, performed as described previously (17), showed radioactivity only in the spots of PC, PDME and PME. The spots were scraped off to measure radioactivity.

RESULTS

Solubilization of PE-dependent methyltransferase activity. The microsomes (10 mg protein/ml) were treated for 30 min on ice with various solubilizing reagents and then the mixtures were centrifuged at 105,000 x g for 60 min at 4 °C and the PE-dependent enzyme activity in the supernatant was measured. Incubation with urea (8 M), even at 24 °C, solubilized only 20% of the total activity, the rest remaining in the precipitate. SDS (0.5%) was more effective, solubilizing almost 70% of the total activity in the microsomes. How-

ever, the solubilized activity was rather unstable and was lost completely on incubation in 0.05% SDS under the assay conditions, and on freezing and thawing twice half the activity of the SDS-treated preparation was lost. DOC (1%) was the most effective of the reagents tested and it solubilized almost all the activity in the microsomes. About one-third of the solubilized activity was lost when PE was not added to the assay mixture. On treatment with Triton X-100 (1%), about 50% of the total activity in the microsomes was solubilized, but in this case three-quarters of the activity was lost in the absence of PE. No increase in specific activity in the supernatant was observed on treatment with any of these reagents. No activity was solubilized with 25 mM phosphate buffer (pH 8.0), 20 mM EDTA or 2 M chaotropic agents, such as NaSCN, guanidine-HCl and Na-perchlorate, and all the activity was recovered in the insoluble material.

Partial purification. In the experiment described above the 0.3% DOC-treated precipitate showed the highest specific activity, and the solubilized fraction did not have a higher specific activity (Fig. 1A,C).

To obtain the PE-dependent methyltransferase system in a soluble form, but with higher specific activity, we first treated the microsomes with 0.3% DOC (10 mg protein/ml) and then solubilized the resulting precipitate (1 mg protein/ml) with Triton X-100. In contrast to the case with intact microsomes (data not shown) with DOC-treated microsomes a concentration of 0.2% Triton X-100 was enough for solubilization of the enzyme system (Fig. 1B,D). Table I summarizes results on the partial purification of the enzyme system. The final preparation is referred to as Triton-enzyme hereafter. Twenty percent of the total PE-dependent activity in the microsomes was recovered in a soluble form in this preparation with 4-fold increase in specific activity. The Triton-enzyme also showed increased PE-dependency.

Properties of Triton-enzyme. The effects of various phospholipids on the Triton-enzyme are shown in Table II. PE, PME, PDME and PC increased methyl incorporation 8.9-, 165-, 79- and 2.1-fold, respectively. The total lipid

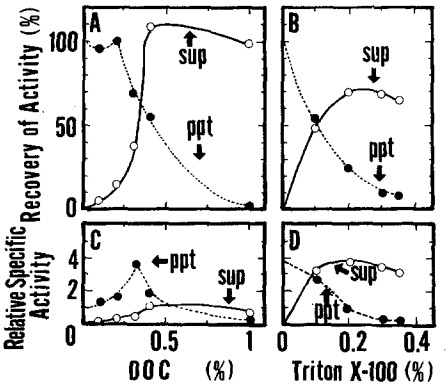

Fig. 1. The enzyme activity was solubilized with DOC and 0.1 M NaCl from intact microsomes (10 mg protein/ml)(A,C), or with Triton X-100 from 0.3% DOC-treated microsomes (1 mg protein/ml)(B,D) as described in the text in a total volume of 1 ml of the buffered solution described in the legend to Table I (*4) but without DOC and NaCl. Then the mixtures were centrifuged and the precipitates were suspended in 1 ml of the same buffer without detergents and NaCl. Activity in the supernatants and precipitates was assayed as described in the MATERIALS AND METHODS in the presence of PE. Activity is shown as a percentage of the incorporation of [^{14}C]methyl groups with intact microsomes(A) and DOC-treated microsomes(B). On treatment of intact microsomes with 0.4% DOC, the total activity recovered in the supernatant and precipitate exceeded 100%. The reason for this is now under investigation. The specific activity is shown relative to that of intact microsomes(C,D).

Table I. Partial Purification of PE-dependent Methyltransferase System for PC Synthesis.

Purification Step	Volume (ml)	Total Protein (mg)	Total Activity*1 (μ mol [^{14}C]methyl incorporated)		Specific Activity*1 (n mol [^{14}C]methyl incorporated/mg)	
			+ None	+ PE	+ None	+ PE
Microsomes	10.7	789	4.96	5.36 (100)*2	6.29	6.79 (1.0)*3
0.3% DOC *4 Treatment	12.7	78.2	1.54	2.31 (43)	19.7	29.5 (4.3)
Solubilization*5 with 0.2% Triton X-100	80.3	40.8	0.12	1.06 (20)	2.92	25.9 (3.8)

*1. Enzyme activity was determined as described in the MATERIALS AND METHODS with (+ PE) and without (+ None) PE.
*2. Relative activity.
*3. Relative specific activity.
*4. Mouse liver microsomes were mixed with DOC (0.3%), NaCl (0.1 M), glycerol (10%), L-cysteine (10 mM) and Na-phosphate buffer, pH 8.0 (10 mM) in a volume of 78.9 ml. (Values in parentheses show final concentrations of chemicals.) The mixtures were stood for 30 min on ice and then centrifuged at 105,000 x g for 60 min.
*5. The DOC-treated precipitates were solubilized on ice for 30 min with 0.2% Triton X-100 in a volume of 80.3 ml of the buffered solution described above, but without DOC and NaCl and then the mixture was centrifuged and the resulting supernatant (Triton-enzyme) was collected.

Table II. Effects of Various Phospholipids on the Activities of the Microsomal Enzyme and Triton-enzyme.

Phospholipid Added	Enzyme Source			
	Intact Microsomes		Triton-enzyme	
None	6.29*1	(1.0)*2	2.92*1	(1.0)*2
PE	6.79	(1.1)	25.9	(8.9)
PME	20.2	(3.2)	482	(165)
PDME	13.1	(2.1)	232	(79)
PC	6.23	(0.99)	6.13	(2.1)
PS	6.84	(1.1)	3.21	(1.1)
CL	5.04	(0.80)	3.50	(1.2)
Total Lipid	7.02	(1.1)	14.6	(5.0)

*1. Incorporation of the [^{14}C]methyl group into phospholipids (n mol/mg/30 min) was assayed as described in the MATERIALS AND METHODS. The phospholipid concentration used, 0.6 mM as phosphate group, gave nearly the maximum activation.
*2. Relative activity.

extracted from the microsomes also increased the incorporation 5.0-fold. These phospholipids had much less effect on the microsomal enzyme.

In the presence of PE the Triton-enzyme formed 83% PC, 13% PDME and 3% PME, whereas the intact microsomes formed 91% PC, 7% PDME and 2% PME. Both preparations showed a pH-optimum at 10.5.

DISCUSSION

In this work we solubilized the PE-dependent methyltransferase system from mouse liver microsomes by successive treatments with DOC and Triton X-100, and found that the specific activity was increased 4-fold by these treatments. The fact that detergents were necessary for solubilization suggests that this enzyme system is at least partly integrated into the membrane. The reaction products with Triton-enzyme were PC, PDME and PME, suggesting that all the methyltransferases in the microsomes were solubilized in our conditions. The finding that PME, PDME and PE all activated the Triton-enzyme also supports this idea, because these compounds are substrates of these methyltransferases.

However, we found that the phospholipid-protein ratio of the Triton-enzyme was almost the same as that of intact microsomes (data not shown). Thus activation of the Triton-enzyme by PE is probably due not only to supply of substrate, but also to a stabilizing effect for maintaining the active structure of the enzyme system and/or protecting it against detergents. The last two possibilities may explain the activation by PC, which is not a substrate of this enzyme system. Conversely the stimulatory effects of PME and PDME may be due mainly by the former possibility, since PME and PDME, which are both metabolic intermediates in PC synthesis from PE, are not present in the microsomes, as described previously (2,18). Phospholipids had effects on the Triton-enzyme but not the transferases in intact microsomes (Table II), suggesting different state of the enzymes and phospholipids in these two enzyme preparations and/or differences in their phospholipid-protein interactions.

Further purification of this enzyme system is now in progress. A preparation that was completely dependent on the presence of PE for activity was obtained by an additional step of purification of the Triton-enzyme (data not shown). Recently Hirata et al. (5,6) demonstrated two methyltransferases in adrenal medulla and erythrocyte membranes that catalyzed a stepwise reaction, and suggested that detergents were necessary for solubilization of the "first" methyltransferase. This suggestion was confirmed in the present work on methyltransferase system in mouse liver microsomes.

The methyltransferase system for the conversion of PE to PC leads to biological transformation of membranes and must be important in the regulation of membrane functions. In this connection it is interesting that this pathway is not present in many transformed cell lines (19,20). Further purification of this enzyme system will be necessary for elucidating its precise reaction mechanism in microsomes, particulary in relation to the interactions and regulatory effects of phospholipids, such as those shown with other membrane enzymes (21).

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REFERENCES

1. Van den Bosch, H. (1974) Ann. Rev. Biochem. 43, 243-277.
2. Bremer, J., and Greenberg, D. M. (1961) Biochim. Biophys. Acta 46, 205-216.
3. Gibson, K. D., Wilson, J. D., and Udenfriend, D. (1961) J. Biol. Chem. 236, 673-679.
4. Scarborough, G. A., and Nyc, J. F. (1967) J. Biol. Chem. 242, 238-242.
5. Hirata, F., Viveros, O. H., Dilberto, E. J., Jr., and Axelrod, J. (1978) Proc. Natl. Acad. Sci. USA 75, 1718-1721.
6. Hirata, F., and Axelrod, J. (1978) Proc. Natl. Acad. Sci. USA 75, 2348-2352.
7. Kaneshiro, T., and Law, J. H. (1964) J. Biol. Chem. 239, 1705-1713.
8. Hirata, F., and Axelrod, J. (1978) Nature 275, 219-220.
9. Rehbinder, R., and Greenberg, D. M. (1965) Arch. Biochem. Biophys. 109, 110-105.
10. Schneider, W. C. (1948) J. Biol. Chem. 176, 259-226.
11. Doi, O., and Nojima, S. (1973) J. Biochem. 74, 667-674.
12. Yang, S. F., Freer, S., and Benson, A. A. (1967) J. Biol. Chem. 242, 477-484.
13. Schroeder, F., Permitter, J. F., Glaser, M., and Vagelos, P. R. (1976) J. Biol. Chem. 251, 5015-5026.
14. Tanaka, Y., Anraku, Y., and Futai, M. (1976) J. Biochem. 80, 821-830.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
16. Ames, B. N., and Dubin, D. J. (1960) J. Biol. Chem. 235, 769-775.
17. Sakakibara, Y., Doi, O., and Nojima, S. (1972) Biochem. Biophys. Res. Comm. 46, 1434-1440.
18. White, D. A. (1973) in Form and Function of Phospholipids (Ansell, G. B., Hawthorne, J. N., and Dawson, R. M. C., Editors), pp. 441-482; Elsevier Scientific Publishing Co., Amsterdam.
19. Figard, P. H., and Greenberg, D. M. (1962) Cancer Res. 22, 361-367.
20. Glaser, M., Ferguson, K. A., and Vagelos, P. R. (1974) Proc. Natl. Acad. Sci. USA 71, 4072-4076.
21. Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 515, 209-237.