Journal of the Korean Chemical Society
1997, *Vol. 41, No. 12*
Printed in the Republic of Korea

Taurodeoxycholate에 의한 뇌 포스포리파제 D의 용해: 및 금속이온의 활성화 효과

崔錫宇 · 崔明彦*
서울대학교 자연과학대학 화학과, 분자촉매 연구센터
(1997. 7. 24 접수)

Solubilization of Brain Phospholipase D by Taurodeoxycholate: Activational Effect of Some Matal Ions

Seok-Woo Choi and Myung-Un Choi*
Department of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-742, Korea
(Received July 14, 1997)

요 약. 쥐의 미크로좀 포스포리파제 D(PLD)를 센 이온세기 상태에서 0.2% taurodeoxycholate를 사용하여 용해시켰다. 포스포리파제 D의 활성은 기질로 방사성 동위원소로 표지 된 dipalmitoylphosphatidylcholine을 사용하여 생성 된 phosphatidic acid(PA)를 측정하여 결정하였다. 용해된 PLD의 최적 pH와 온도는 각각 6.5와 30 °C로서 용해되기 전 미크로좀 상태의 PLD와 비슷하였다. 올레산의 활성화 효과는 4 mM 농도에서 관찰되었다. PLD 활성도에 미치는 금속이온 영향을 조사한 결과 Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}와 같은 알칼리 토금속은 모두 PA 생성을 촉진시킨 반면, Cu^{2+}, Cd^{2+}, Al^{3+}, Ni^{2+}, V^{5+}는 억제하였다.

ABSTRACT. Microsomal phospholipase D (PLD) in rat brain was solubilized employing 0.2% taurodeoxycholate in high ionic strength. Phopholipase D activity was determined by measuring product phophatidic acid (PA) using isotope-labelled dipalmitoylphophatidylcholine as a substrate. The solubilized PLD showed an optimal pH of 6.5 and the highest activity at 30 °C. These properties were similar to those of microsomal PLD before solubilization. The stimulatory effect of oleic acid was observed at the concentration of 4 mM. When effects of metal ions on PLD activity were examined, alkaline earth metals such as Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} promoted the PA production but Cu^{2+}, Cd^{2+}, Al^{3+}, Ni^{2+}, V^{5+} showed inhibitory effects.

INTRODUCTION

Phospholipase D (PLD) acts to catalyze the hydrolysis of phospholipids to yield phosphatidic acid (PA) and the corresponding head groups.[1] PLD activity has been identified in a wide variety of cell types and organisms from bacteria, yeast, plants, and mammals.[2] The product PA is thought to act as an effector for a broad spectrum of physiological processes including secretion, DNA synthesis, and cell proliferation.[3] Therefore cellular PLD is emerging as one of major component in receptor-coupled signal transduction.[4] Numerous studies demonstrated that activation of PLD in mammals occurred via several pathways involving protein kinase C, heterotrimeric GTP-binding proteins, or small G proteins of the ARF and Rho families.[5] Recently genes encoding PLD have been published for castor bean, rice, human, and yeast.[6]

In spite of the wealth of report concerning the possible function of PLD, only limited information is available about the molecular properties of PLD because of slow progress in obtaining purified PLD. A major obstacle to purification of PLD probably due to the membrane-bound nature of PLD and the

multiplicity of activation pathways involved in PLD.[4] Furthermore in vitro assay conditions for PLD are complicated by interfacial interaction of substrate phospholipid with the enzyme protein like other phospholipases.[7] Therefore the PLD activities observed from many mammalian tissues and cell lines differ each other in their subcellular localization,[8-11] pH optima,[10,12] dependence of divalent cation,[8,10] activation factors such as phosphatidylinositol 4,5-bisphosphate (PIP_2),[5] ADP-ribosylation factor (ARF),[5] and oleate.[9] In order to resolve these issues of ambiguities of PLD properties, many groups have attempted to solubilize the particulated PLD from various sources and tried to isolate homogenous PLD. Since Taki and Kanfer tried to purify PLD from rat brain using detergent Miranol in 1979,[8] various types of detergent were examined including Triton X-100.[13,14] Recently two major forms of PLD activity, oleate-dependent and ARF-dependent, were resolved by HPLC on a heparin-5PW column containing octylglucoside.[16] The ARF-dependent PLD was also partially purified from cholate extracted PLD of porcine brain.[17]

In this study, taurodeoxycholate, another anionic detergent, was examined its effectiveness of solubilization capability on microsomal PLD in rat brain. The taurodeoxycholate-solubilized PLD was partly characterized including metal ion effects.

EXPRIMETAL

Materials. 1,2-di[1-^{14}C]palmitoyl-L-3-phosphatidylcholine ([^{14}C]PC, 112 μCi/μmol) was purchased from Amersham (Aylesbury, U.K.). Sodium oleate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2-[N-morpholino]ethanesulfonic acid (MES) and taurodeoxycholic acid (TDC) were obtained from Sigma Co. (St. Louis, U.S.A.). Precoated silica gel TLC plate (Kieselgel 60 F254) was obtained from Merck (Darmstadt, Germany). Crude egg phosphatidylcholine (PC) from Sigma was purified by aluminum oxide column (neutral type). Phosphatidic acid (PA) was prepared from PC using cabbage PLD according to the procedure described previously.[18]

Preparation of microsomal fraction. Wistar rats (female, 4 week-old) were sacrificed by decapitation and whole brains were homogenized in 10 volumes of 0.32 M sucrose solution. Nuclei and unbroken cells were sedimented at 10,000 g for 10 min and the resultant supernatant was centrifuged at 100,000 g for 60 min to obtain microsomal fraction.

Solubilization of microsomal PLD. Solubilization was routinely performed in an ice-water bath. The microsomal fraction was suspended with 2 ml HEPES buffer (pH 7.0) solution containing 500 mM NaCl and 0.2% taurodeoxycholate and then sonicated with ultrasonic microtip for 10 times in pulsed mode at the intensity of 2.0. The mixture was incubated at 4 °C for 30 min with several mild vortexing and then centrifuged at 100,000 g for 60 min to obtain the solubilized protein extract. An aliquot of 25 μl of this extract was used for the assay of solubilized PLD activity.

Assay of phospholipase D. The standard reaction mixture consisted of 0.4 mM PC with 0.1 μCi [^{14}C]PC (0.9 nmol), 20 mM $CaCl_2$, 25 mM KF, 5 mM sodium oleate, 40 mM HEPES buffer (pH 7.0) and 25 μl protein (about 70-400 μg) in total volume of 100 μl. PC and sodium oleate were lyophilized in microcentrifuge tube by a stream of nitrogen gas. The reaction mixture after added HEPES buffer containing other reagents was sonicated for 1 min in a bath-type sonicator. The reaction was started by addition of 25 μl of the enzyme source and incubated for 90 min at 30 °C. The reaction mixture of zero time incubation was taken as the blank. The reaction was terminated by addition of 1 ml of chloroform: methanol (2:1) solution. The extracted phospholipids with carrier PA were separated on TLC plated employing an organic solvent system (chloroform:methanol:acetone:acetic acid:water=50:15:15:10:5 by volume). The spot of PA (R_f=0.62) was identified by staining the plate with iodine vapor and was scraped off into vial for quantification by liquid scintillation counting with an efficiency of 85%. Protein concentration was measured by the method of Bradford[19] using ovalbumin as a standard. Phospholipids was determined by the method of Bartlett.[20]

RESULTS AND DISCUSSION

Solubilization of microsomal PLD. Detergent and salt are known to be critical for solubilizations of membrane-bound proteins. In a series of experiments to search for proper detergent for solubilization of brain PLD, we found that taurodeoxycholate (TDC) has a stimulatory effect on the PLD in brain. Therefore the effect of taurodeoxycholate on the PLD activity was examined in the condition of solubilization (*Fig.* 1). The PLD activity showed maximum at concentration of 0.05% TDC in both solubilized supernatant and pellet fraction. This activational effect of anionic bile salt was similar to previous report.[21] For solubilization of microsomal PLD, various concentration of TDC was examined in the range of 0.1-0.8% (*Fig.* 2). The solubilized PLD activity was fairly lost in higher concentration over 0.2% TDC, even though the amount of solubilized protein was increased. This implies that the concentration of TDC in solubilization is very critical to retain the PLD activity as well as for the activation of PLD. Therefore the PLD assay had to be performed uniformly in the presence of 0.1% TDC. At 0.1-0.4% TDC, the PLD activity in solubilized fraction was

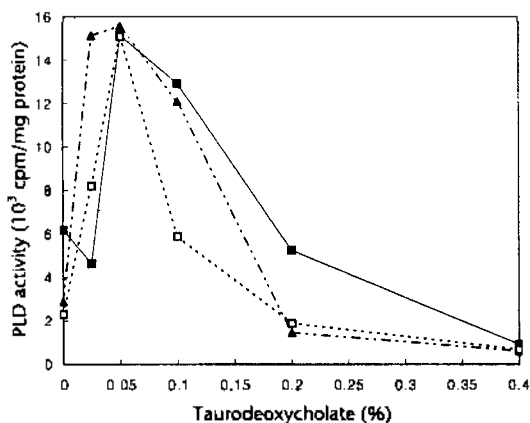

Fig. 1. Effect of taurodeoxycholate on PLD activity. PLD activity of 1000 cpm/ mg protein is equivalent to 0.140 nmol PA/ mg protein/hr. The solubilization medium contained 500 mM NaCl and the salt was diluted to 1/4 in assay mixture. (▲) original suspension of microsome; (■) solubilized supernatant; (□) resuspended pellet.

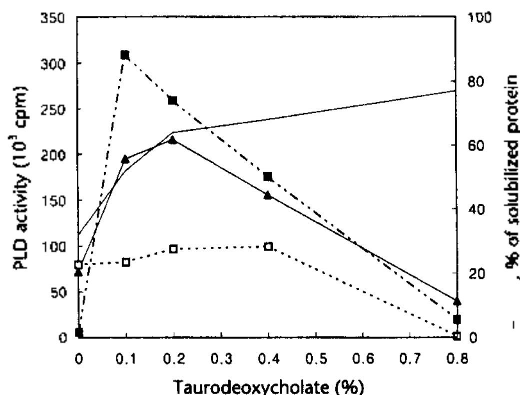

Fig. 2. Solubilization of PLD with taurodeoxycholate. All solubilizing medium contained 500 mM NaCl. Each assay was performed at final concentration of 0.1% TDC and 125 mM NaCl. (▲) original suspension of microsome; (■) solubilized supernatant; (□) resuspended pellet.

higher than in original suspension of microsomes. Thus the ratio of solubilization could not be estimated in terms of a total solubilized activity relative to original suspension of the microsomal activity, which is well above 100% at 0.2% TDC. At this concentration, however the solubilization ratio could be estimated as 70% in terms of total activity present in supernatant relatively to the sum of the activity of solubilized supernatant and pellet. When the effect of NaCl on solubilization of PLD was examined with various concentration of salt at 0.2% detergent concentration, it was apparent that solubilization increased gradually up to 1,500 mM NaCl (data not shown). However the 500 mM NaCl seemed to be more proper concentration than 1,500 mM in respect to column works for purification as well as recovery of the solubilized enzyme. Nonetheless the ionic strength of the solubilization medium is critical, because interaction between membrane-bound proteins can be polar (e.g. ionic interactions) as well as non-polar. The ionic interaction was reduced with increasing salt concentration leading to a more effective dissociation of membrane proteins from soluble molecules as well as from other non-soluble membrane constituents.

Properties of solubilized PLD. The pH dependence of solubilized PLD was shown in *Fig.* 3A.

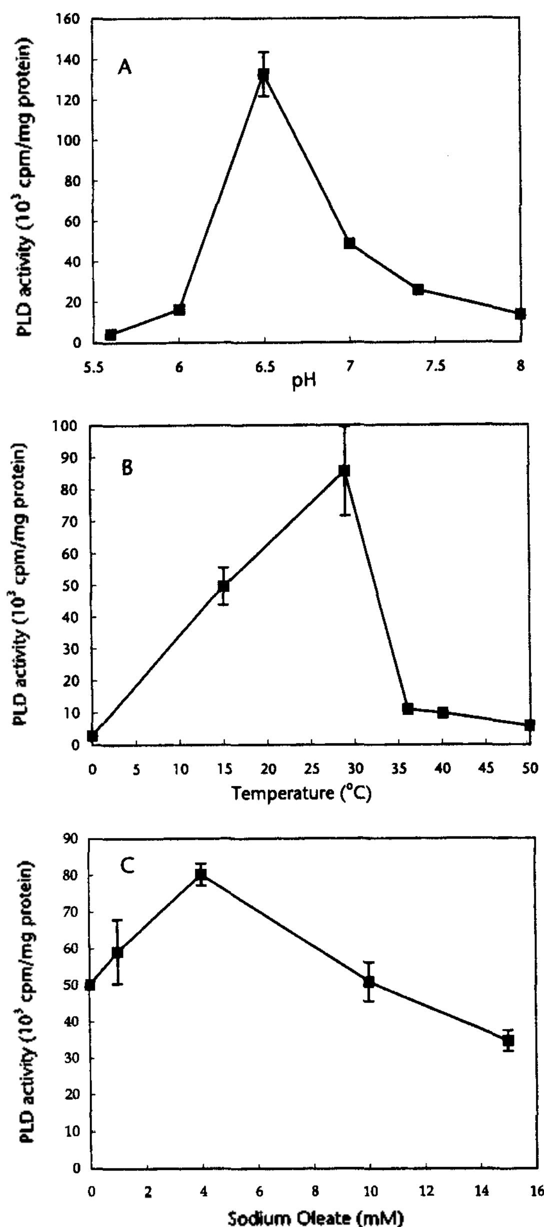

Fig. 3. Properties of solubilized PLD. (A) MES buffer (100 mM) for the pH range of 5.6 to 6.5 and HEPES buffer (100 mM) for the range of 7.0 to 8.0 were employed. (B) Effect of temperature. (C) Effect of sodium oleate. Reaction was carried out with 130 μg protein.

The solubilized PLD has the optimal activity at pH 6.5. When effect of incubation temperature on the enzyme activity was investigated, its optimal temperature was found to be around 30°C (*Fig.* 3B). These properties were similar to that of microsomal PLD before solubilization (data not shown). Sodium oleate, which was used as an activator for the oleate-dependent PLD assay, was examined its effectiveness on the solubilized PLD activity (*Fig.* 3C). The optimal concentration of oleate was found to be 4 mM. This concentration agreed with that of microsomal PLD before solubilization even the quantity of solubilized protein used in the assay was one third of the microsomal protein. It has been demonstrated previously that optimal activation by oleate was influenced by the molar ratios with microsomal protein and PC substrate.[21] Hence the reported optimal value of 4 mM of oleate per 100 μg of solubilized protein is likely to correspond to 300 μg of microsomal protein.

Metal ion effect. Effects of various metal ions on the activity of solubilized PLD were examined (*Table* 1). All metals examined had chloride base. Cu^{2+}, Cd^{2+}, Al^{3+}, Ni^{2+} and V^{5+} inhibited the PA production, while Li^{+}, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} showed some stimulatory effects. It is noticeable that all of alkaline earth metal ions tested stimulated the PA production. Especially, Ca^{2+} and Ba^{2+} showed unusually high degree of stimulatory effects. In the case of partially purified PLD of rat

Table 1. Effects of various metal ions on the solubilized PLD activity of rat brain

Metal ion	PA production	
	nmol/mg protein/h	ratio[a]
EDTA	0.31	1.00
Cu^{2+}	0.08	0.24
Cd^{2+}	0.09	0.30
Al^{3+}	0.11	0.34
Ni^{2+}	0.12	0.37
V_2O_5	0.20	0.66
Li^{+}	0.40	1.28
Mg^{2+}	2.16	6.91
Ca^{2+}	4.59	14.66
Sr^{2+}	3.34	10.67
Ba^{2+}	8.94	28.59

The concentration of all metal ions except V_2O_5 was 15 mM in the presence of 5 mM of pre-added EDTA. [a]These values were compared with that of reaction containing only EDTA.

brain Mg^{2+} did not stimulate the PLD activity, because probably due to an absence of PKC in purified state.[8] In many cell types, the dependency of PLD activity on Ca^{2+} ion have been shown frequently,[22,23] whereas stimulation by barium ion has been observed only one case.[24,25] When concentration dependency of calcium and barium ions on the solubilized PLD activity were investigated, both ions promoted the PA production in a concentration dependent manner (data not shown). At 30 mM of calcium and barium ions, PLD activities were observed to be 17 and 30 nmol/mg protein/hr, respectively. While the effect of alkaline earth metal ions on promoting the PLD activity was evident, elucidation of molecular mechanism for the metal effects definitely requires further studies.

Acknowledgement. This work was supported in part by research grants from Korea Science and Engineering Foundation (92-2400-04-01-3) and through the Center for Molecular Catalysis at Seoul National University.

REFERECES

1. (a) Horwitz, J.; Davis, L. L. *Biochem. J.* **1993**, *295*, 793. (b) Holbrook, P. G.; Pannell, L. K.; Daly, J. W. *Biochim. Biophys. Acta* **1991**, *1084*, 155. (c) Kim, B.; Choi, M. *Prog. Chem. & Chem. Eng.* **1991**, *31*, 952.
2. Heller, M. *Adv. Lipid Res.* **1978**, *16*, 267.
3. (a) Boarder, M. R. *Tips* **1994**, *15*, 57. (b) Thompson, N. T.; Garland, L.; Bouser, R. W. *Adv. Pharmacol.* **1993**, *24*, 199. (c) Cuadrado, A.; Carnero, A.; Dolfi, F.; Jimenez, B.; Lacal, J. C. *Oncogene* **1993**, *8*, 2959.
4. (a) Exton, J. H. *Biochim. Biophys. Acta* **1994**, *1212*, 26. (b) Liscovitch, M. *Signal-activated phospholipases* **1994**, R.G. Landes Co. Austin. (c) Divecha, N.; Irvine, R. *Cell* **1995**, *80*, 269.
5. (a) Brown, H. A.; Gutowski, S.; Moormaw, C. R.; Slauter, C.; Sternweis, P. C. *Cell* **1993**, *75*, 1137. (b) Han, J.-S.; Chung, J.-K.; Kang, H.-S.; Donaldson, J.; Bae, Y. S.; Rhee, S. G. *J. Biol. Chem.* **1996**, *271*, 11163.
6. (a) Wang, X.; Xu, L.; Zheng, L. *J. Biol. Chem.* **1994**, *269*, 20312. (b) Veki, J.; Morika, S.; Komari, L.; Kumashiro, T. *Plant Cell Physiol.* **1995**, *36*, 903. (c) Hammond, D. M.; Altshuller, Y. M.; Sung, T.-C.; Rudge, S. A.; Rose, K.; Engebrecht, J.; Morris, A. J.; Frohman, M. A. *J. Biol. Chem.* **1995**, *270*, 29640. (d) Waksman, M.; Eli, Y.; Liscovitch, M.; Gerst, J. E. *J. Biol. Chem.* **1996**, *271*, 2361.
7. Dennis, E. A. *Methods in Enzymol.* **1991**, *197*, Section I.
8. Taki, T.; Kanfer, J. N. *J. Biol. Chem.* **1979**, *254*, 9761.
9. Kobayashi, M.; Kanfer, J. N. *J. Neurochem.* **1987**, *48*, 1597.
10. Chalifa, V.; Mohn, H.; Liscovitch, M. *J. Biol. Chem.* **1990**, *265*, 17512.
11. Wang, P.; Anthes, J.; Siegel, M.; Egan, R. W.; Billah, M. M. *J. Biol. Chem.* **1991**, *266*, 14877.
12. Hattori, H.; Kanfer, J. N. *J. Neurochem.* **1985**, *45*, 1578.
13. Kobayashi, M.; Kanfer, J. N. *Methods in Enzymol.* **1991**, *197*, 575.
14. Kanoh, H.; Kanaho, Y.; Nozawa, Y. *Lipids* **1991**, *26*, 426.
15. Massenburg, D.; Han, J. -S.; Liyanage, M.; Patton, W. A.; Rhee, S. G.; Moss, J.; Vaughan, M. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11718.
16. Okamura, S.; Yamashita, S. *J. Biol. Chem.* **1994**, *269*, 31207.
17. Brown, H. A.; Gutowski, S.; Kahn, R. A.; Sternweis, P. C. *J. Biol. Chem.* **1995**, *270*, 14935.
18. Jung, K.; Koh, E.; Choi, M. *Bull. Korean Chem. Soc.* **1989**, *10*, 595.
19. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
20. Bartlett, G. R. *J. Biol. Chem.* **1959**, *234*, 466.
21. Chalifour, R; Kanfer, J. N. *J. Neurochem.* **1982**, *39*, 299.
22. Balsinde, J.; Diez, E.; Mollinedo, F. *Biochem. Biophys. Res. Commun.* **1988**, *154*, 502.
23. Billah, M. M.; Pai, J. -K.; Mullamann, T. J.; Siegel, M. I. *J. Biol. Chem.* **1989**, *264*, 9069.
24. Kanfer, J. N.; McCartney, D. *FEBS Lett.* **1994**, *337*, 251.
25. Liscovitch, M.; Chalifa-Caspi, V. *Chem. Phys. Lipids* **1996**, *80*, 37.