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Inhibition of *Streptomyces chromofuscus* Phospholipase D Activity by Dichloro-(2,2′:6′,2″-terpyridine)-platinum (II) Dihydrate

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To determine the catalytic site of *Streptomyces chromofuscus* phospholipase D (PLD), which lacks an HKD motif, we examined the effects of inhibitors on the hydrolytic activity of the PLD by comparing it with cabbage and *Streptomyces* PLDs, which have two HKD motifs. We showed that dichloro-(2,2′:6′,2″-terpyridine)-platinum (II) dihydrate, a His- and Cys-directed chemical modifier, had inhibitory effects on the activities of all types of PLD examined. On the other hand, *N*-ethylmaleimide, a thiol-directed modifier had no such effects on PLD activity. These results suggest that the His residue plays an important role in the activity of *Streptomyces chromofuscus* PLD.

Keywords: Phospholipase D; *Streptomyces*; dichloro-(2,2′:6′,2″-terpyridine)-platinum (II) dihydrate

INTRODUCTION

Phospholipase D (EC3.1.4.4., PLD), which can be obtained from mammals, plants, and bacteria, catalyzes the hydrolysis of the ester bond between the phosphatidic acid and alcohol moieties of phospholipids. Additionally, this enzyme catalyzes the transphosphatidylation reaction in the presence of alcohols as a nucleophile donor. Several PLDs have been cloned and sequenced, and they could be divided into two classes according to their primary structures.

One class comprises almost all PLDs from mammals, plants, yeast and bacteria; PLDs in this class contain two separate copies of the highly conserved motif "HxKxxxxD", so-called HKD motif, in their structures.[1–8] The endonuclease nuc, which is a member of the superfamily of PLDs with two HKD motifs, was characterized and found to contain only one conserved HKD motif. Gottlin *et al.* have reported that the His residue of nuc plays a key role in catalysis via a covalent phosphohistidine intermediate.[8] Recently, the deletion mutants of PLD that contain only an N- or a C-terminal HKD domain have been reported to exhibit no catalytic activity in rat brain PLD1[9] and PLD from *Streptomyces* sp.[10] In addition, when the N- and C-terminal halves of PLD coexist, two fragments physically associate and the catalytic activity of the enzyme is restored.[9,10] Therefore, it is presumed that two HKD motifs are required for PLD activity.

On the other hand, *Strptomyces chromohuscus* PLD is characterized by the lack of an HKD motif,[11] and has been investigated in terms of its activation mechanism by phosphatidic acid.[12–14] However, no definite information is available on the catalytic site of this enzyme.

In this study, we investigated the *Strptomyces chromohuscus* PLD, by comparing it with cabbage and *Streptomyces* PLDs with two HKD motifs to gain more information about its catalytic site. We first examined the effect of a His- and Cys-directed chemical modification agent, dichloro-(2,2′:6′,2″-terpyridine)-platinum (II) dihydrate (DTPD, Figure 1), on the hydrolytic activities of the three mentioned PLDs spectrophotometrically using phosphatidyl-*p*-nitrophenol (PpNP) as an artificial substrate.

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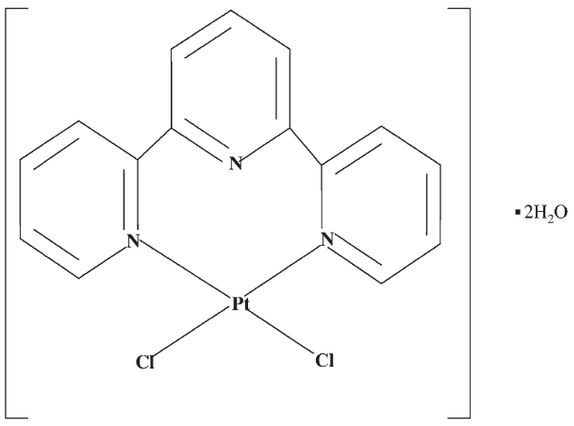

FIGURE 1 The structure of DTPD.

MATERIALS AND METHODS

Materials

Cabbage PLD (Type I) was obtained from Sigma. *Streptomyces chromofuscus* PLD (Type VI) and *Streptomyces* PLD (Type VII) were kindly provided by Asahi Chemical Industry. Type I and VII PLDs have two HKD motifs, whereas Type VI has no motifs.[6,7,11] DTPD was purchased from Aldrich Co. A PD-10 column was purchased from Amersham Pharmacia Biotech. *N*-Ethylmaleimide (NEM) was purchased from Wako Pure Chemical Industries Ltd. PpNP was synthesized chemically as described previously[15] using phosphatidic acid from soybean, which was a gift from Kao Co. All other chemicals were purchased from commercial sources and were of the highest grade.

Enzyme Assay

The procedure was performed basically following the previously reported method[15] except for the reaction temperature and PpNP concentration. In this study, the hydrolysis reaction of PpNP (2 mM) with phosphatidic acid and *p*-nitrophenol was performed at 37°C for 10 min in 0.1 M acetate buffer pH 5.0. Unless otherwise noted, all enzymatic assays were performed at pH 5.0. The reaction was stopped by heating at 90°C for 30 s, and the mixture was diluted tenfold with 0.1 M Tris buffer pH 8.0. The liberated *p*-nitrophenol was monitored based on its absorbance at 405 nm (molecular extinction coefficient of *p*-nitrophenol; $\varepsilon = 18,450$).[15] One unit of PLD was defined as the amount that released 1 μmol of *p*-nitrophenol per min under the assay conditions.

DTPD Treatment

DTPD was added to each sample of PLD (Type I: 0.11 U/sample, Type VI: 0.68 U/sample, and Type VII: 0.79 U/sample), and the mixture was then incubated at room temperature for 45 min. Following incubation, excess DTPD was removed using a PD-10 column. Then, 5 mM His (pH 7.4) was added to the mixture to react with any residual DTPD.

NEM Treatment

We next examined the involvement of a Cys residue in the enzyme's activity using NEM, a thiol-specific modification agent. NEM labeling was carried out using the previously reported method.[16] PLDs (Type I 2.0 U/sample, Type VI: 3.6 U/sample, and Type VII: 4.0 U/sample) were pre-incubated with 1 mM NEM in 30 mM Tris–HCl (pH 7.0) at room temperature. The labeling was terminated after 2 min by adding dithiothreithol to a final concentration of 5 mM.

Aluminum Fluoride (AlF_4^-) Inhibition Assay

PLDs (Type I: 0.11 U/sample, Type VI: 0.14 U/sample, and Type VII: 0.20 U/sample) were pre-incubated with 3 mM sodium fluoride (NaF), 20 μM aluminium chloride ($AlCl_3$) or 3 mM NaF + 20 μM $AlCl_3$ (AlF_4^-) at 37°C for 10 min in 10 mM Tris–HCl buffer (pH 7.4).

RESULTS

Basic Properties of PLDs

The optimum pH for activity of each PLD was 5.0 (data not shown).

The Ca^{2+} requirement for the hydrolytic activities of PLDs was examined by adding EDTA at various concentrations to the enzymatic reaction mixture containing 10 mM Ca^{2+} (Figure 2). The activity of Type VII PLD was found to be independent of Ca^{2+}.

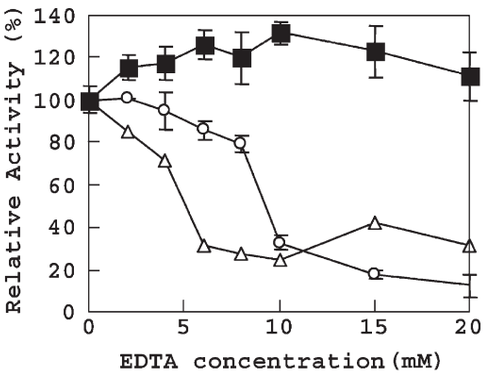

FIGURE 2 Ca^{2+} requirement for hydrolytic activities of PLDs. Type I (Δ: 0.11 U/sample), VI (○: 0.68 U/sample), VII (■: 0.79 U/sample) PLDs were assayed by the hydrolysis reaction at 37°C in a mixture of 2 mM PpNP, 10 mM Ca^{2+} and EDTA at various concentrations in 0.1 M acetate buffer (pH 5.0) for 10 min.

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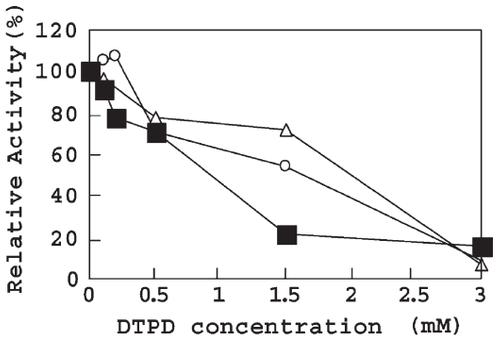

FIGURE 3 Concentration-dependent inhibition of PLD activity by DTPD modification. Type I (Δ: 0.111 U/sample), VI (○: 0.68 U/sample), VII (■: 0.79 U/ sample) PLDs were incubated with DTPD in 20 mM Tris–HCl buffer (pH 7.4) for 45 min at room temperature.

On the other hand, Ca^{2+} was found to be essential for the activity of Type I and VI PLDs. Our results for Type VI and VII PLDs regarding their Ca^{2+} requirement were the same as those of a previous study.[17] From this result, the assay was performed using 10 mM Ca^{2+}.

The Michaelis–Menten constants (*K*m) toward PpNP were determined to be 0.81 mM for Type VI PLD and 1.05 mM for Type VII PLD, as determined by the analysis of [S]/v *vs* [S] plot. Type I PLD was assayed similarly, but the *K*m value was not calculated because the enzyme has low affinity toward PpNP.

Effect of DTPD on Activities of Type I, VI and VII PLDs

DTPD inhibited the activities of Type I, VI and VII PLDs in a dose-dependent manner (Figure 3). The inactivation was time-dependent and the inhibition rates became constant about 1.5 h after DTPD addition (Figure 4).

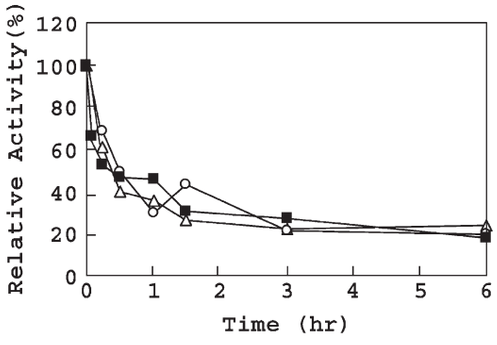

FIGURE 4 Time-dependent inhibition of PLD activity by DTPD modification. Type I (Δ: 0.11U /sample), VI (○: 0.68 U/sample), VII (■: 0.79 U/sample) PLD was incubated with 3, 1 or 3.5 mM DTPD in 20 mM Tris–HCl buffer (pH 7.4) respectively at room temperature.

Effects of NEM on Activities of Type I, VI and VII PLDs

Although DTPD is more selective than other His-directed acylating agents, it can modify a Cys residue.[18] NEM had no effects on the activities of Type I, VI and VII PLDs. The activities of Type I, VI and VII PLDs, which were presented as a % of control, were 101.7 ± 3.1, 99.8 ± 2.4 and 100.2 ± 1.8 (means ± S.D., n = 3) respectively.

DISCUSSION

His residues tend to function as proton shuttle residues in attaining proton-dissociation equilibrium (p*K*a 6.3 ~ 6.6) at neutral pH.[19] In addition, considering the involvement of the HKD motif, we first examined the His-directed chemical modification of PLD activities. However, typical His-directed chemical modifiers are not specific only to His residues. For example, diethylpyrocarbonate, generally used as a His-modifier also modifies Cys, Tyr and Lys residues, although it is more specific than other acylating agents.

In several studies, DTPD has been used to modify His residues,[18,20] and it was found that Type I, VI and VII PLDs were inhibited by DTPD in a dose- and time-dependent manner (Figures 3 and 4). However, in the study of the interaction between DTPD and short peptides, DTPD was also found to react with a free Cys residue.[18] Type I, VI and VII PLDs have eight, one and eight Cys residues, respectively, in their primary structures.[6,7,11] Therefore, to ensure that the inactivation of PLD by DTPD is due to His modification, we next examined the effect of NEM, a thiol modificator, on PLD activity. NEM had no effects on the activities of the three PLDs. This result suggests that Cys residues in the PLDs are not related to the catalytic center.

It has been reported that AlF_4^-, a phosphate analog, inhibits the activities of PLDs from cabbage and rat through a mechanism of action based on its phosphate-mimicking property.[21,22] AlF_4^- is considered to directly interact with the HKD motif, which is highly conserved among PLD superfamily. Therefore, to confirm this, we examined the effect of AlF_4^- on the activities of Type I, VI and VII PLDs. The presence of AlF_4^- (added as a combination of 3 mM NaF and 20 μM $AlCl_3$) potently inhibited the Type I PLD activity to 25% of the control (Figure 5A). This result agreed with that of a previous report.[21] On the other hand, AlF_4^- had no effect on the activities of Type VI and VII PLDs (Figures 5B and C). This result suggests that AlF_4^- has a different mode of action against Type VI and VII PLDs from that against Type I

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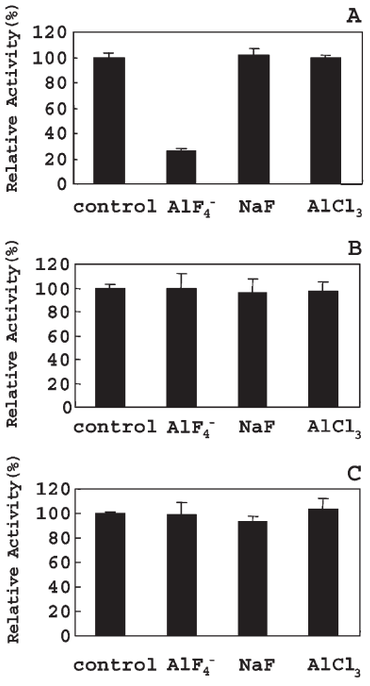

FIGURE 5 Effects of AlF_4^- on hydrolytic activities of PLDs. A: Type I PLD (0.11 U/sample). B: Type VI PLD (0.14 U/sample). C: Type VII PLD (0.20 U/sample). The enzymes were pre-incubated with 3 mM sodium fluoride (NaF), 20 μM aluminum chloride ($AlCl_3$) or 3 mM NaF + 20 μM $AlCl_3$ (AlF_4^-) in 10 mM Tris–HCl buffer (pH 7.4) for 10 min at room temperature. Values are means ± S.D., $n = 3$.

PLD, and that AlF_4^- does not directly interact with the HKD motif.

In conclusion, the His residue plays a key role in the activity of *Streptomyces chromofuscus* PLD, which has fourteen His residues in its primary structure.[11] Further experiments are needed to clarify which His residue predominantly contributes to the activity of *Streptomyces chromofuscus* PLD.

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References

[1] Hammond, S.M., Altshuller, Y.M., Sung, T.C., Rudge, S.A., Rose, K., Engebrecht, J., Morris, A.J. and Frohman, M.A. (1995) *J. Biol. Chem.* **270**, 29640.
[2] Ponting, C.P. and Kerr, I.D. (1996) *Protein Sci.* **5**, 914.
[3] Morris, A.J., Engebrecht, J. and Frohman, M.A. (1996) *Trends Pharmacol. Sci.* **17**, 182.
[4] Koonin, E.V. (1996) *Trends Biochem. Sci.* **21**, 242.
[5] Zhao, Y., Stuckey, J.A., Lohse, D.L. and Dixion, J.E. (1997) *Protein Sci.* **6**, 2655.
[6] Iwasaki, Y., Nakano, H. and Yamane, T. (1994) *Appl. Microbiol. Biotechnol.* **42**, 290.
[7] Kim, D., Roh, T., Lee, J., Noh, J., Jang, Y., Hoe, K., Yoo, H. and Choi, M. (1999) *Biochim. Biophys. Acta* **1437**, 409.
[8] Gottlin, E.B., Rudolph, A.E., Zhao, Y., Matthews, H.R. and Dixon, J.E. (1998) *Proc. Natl Acad. Sci. USA* **95**, 9202.
[9] Xie, Z., Ho, W.T. and Exton, J.H. (1998) *J. Biol. Chem.* **273**, 34679.
[10] Iwasaki, Y., Horiike, S., Matushima, K. and Yamane, T. (1999) *Eur. J. Biochem.* **264**, 577.
[11] Yoshioka, I., Mizoguchi, M., Takahara, M., Imamura, S., Beppu, T. and Horinouchi, S. (1992) Japanese Patent No. H3-187382.
[12] Dong, G., Chura, J. and Roberts, M.F. (1998) *J. Biol. Chem.* **273**, 12195.
[13] Dong, G., Baker, D.P., Foley, S.F., Zhou, C., Stieglitz, K. and Roberts, M.F. (1999) *Biochim. Biophys. Acta* **1430**, 234.
[14] Kim, S., Seaton, B. and Roberts, M.F. (1999) *J. Biol. Chem.* **274**, 35367.
[15] D'Arrigo, P., Piergianni, V., Scarcelli, D. and Servi, S. (1995) *Anal. Chim. Acta* **304**, 249.
[16] Pas, H.H. and Robillard, G.T. (1988) *Biochemistry* **27**, 5515.
[17] Carrea, G., D'Arrigo, P., Piergianni, V., Roncaglio, S., Secundo, F. and Servi, S. (1995) *Biochim. Biophys. Acta* **1255**, 273.
[18] Brothers, H.M. and Kostic, N.M. (1988) *Inorg. Chem.* **27**, 1761.
[19] Supuran, C.T. and Scozzafava, A. (2001) *Curr. Med. Chem.-Imm., Endoc. Metab. Agents* **1**, 61.
[20] Mooney, B.P., David, N.R., Thelen, J.J., Miernyk, J.A. and Randall, D.D. (2000) *Biochem. Biophys. Res. Comm.* **267**, 500.
[21] Li, L. and Fleming, N. (1999) *FEBS Lett.* **461**, 1.
[22] Li, L. and Fleming, N. (1999) *FEBS Lett.* **458**, 419.

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