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Articles

Evidence for an Essential Lysyl Residue in Phospholipase D from *Streptomyces* sp. by Modification with Diethyl Pyrocarbonate and Pyridoxal 5-Phosphate

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ABSTRACT: Diethyl pyrocarbonate inactivated phospholipase D from *Streptomyces* PMF with second-order rate constants of 0.7 M^{-1} s^{-1} at pH 6.1 or 222 M^{-1} s^{-1} at pH 8.3 and 25 °C, and modified 5 His residues per enzyme molecule. The His residues, however, were not essential for activity because: (a) the second-order rate constants for reaction of diethyl pyrocarbonate with the His residues of the enzyme, which were 1.4 M^{-1} s^{-1} at pH 6.1 or 7.2 M^{-1} s^{-1} at pH 8.3 and 25 °C, differed, both at low and high pH values, from the inactivation rates, and (b) the reversal of His modification by hydroxylamine was not accompanied by recovery of activity. As demonstrated by dinitrophenylation experiments carried out on the treated enzyme, diethyl pyrocarbonate also modified up to 20 Lys residues per enzyme molecule. Other amino acid residues and the conformation and hydrodynamic volume of the enzyme were not modified. The involvement of a Lys residue in enzyme activity was confirmed through experiments with pyridoxal 5-phosphate which inactivated phospholipase D, after $NaBH_4$ reduction, with a second-order rate constant of 3.5 M^{-1} s^{-1} at pH 8.5 and 15 °C. The inactivation took place with concomitant modification of 4 Lys residues, only one of which was found to be essential using the kinetic method of Tsou (Tsou, C.-L. (1962) *Sci. Sin. 11*, 1535−1538). Dicaproyl phosphatidylcholine markedly protected the enzyme against inactivation by DEP or PLP, and this strongly suggests that the essential Lys residue is located in or near the substrate binding site.

The phospholipases D (EC 3.1.4.4), which can be obtained from mammals, plants, and bacteria (Dennis, 1991), are enzymes of remarkable practical importance. In the presence of an alcohol as nucleophile donor, they can catalyze the preparative-scale transphosphatidylation of the naturally abundant phosphatidylcholine to a large group of natural or unnatural phospholipids of interest to pharmaceutical and food industries (Van Nieuwenhuyzen, 1981; Juneja et al., 1987; D'Arrigo et al., 1994). The extent of the competing hydrolytic reaction depends, among other factors, on the source of the enzyme (Juneja et al., 1988). Recently, we have reported the purification of two PLDs[1] from *Streptomyces* sp. which have high transphosphatidylation activity and selectivity (Carrea et al., 1995).

For other phospholipases, such as group I, II, and III phospholipase A_2, the mechanism of action has been elucidated and shown to be based on the concerted action of a His and an Asp which polarize a bound H_2O, making it able to attack the carbonyl group (Dennis, 1994). Instead, PLD's mechanism is far from clarified in detail. Stereochemical studies strongly suggest that, in analogy to serine

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[1] Abbreviations: PLD, phospholipase D; DEP, diethyl pyrocarbonate; FDNB, 1-fluoro-2,4-dinitrobenzene; PLP, pyridoxal 5-phosphate; DCPC, dicaproyl phosphatidylcholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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proteases, a phosphatidyl-enzyme intermediate is formed initially, which can then be transphosphatidylated by reaction with an alcohol to form a new phospholipid or hydrolyzed to phosphatidic acid (Bruzik & Tsai, 1984). However, almost nothing is known about the amino acid residues involved in the catalytic mechanism, also because no tridimensional structure is available for any PLD. To our knowledge, the only studies aimed at detecting the PLD residues essential for activity involved chemical modification of PLD from cabbage and indicated a Cys and a His residue in the active site (Lee et al., 1989), or were done with a mutagenized enzyme from *Corynebacterium pseudotuberculosis*, and these suggested the presence of an essential His (Haynes et al., 1992).

In the present study we investigated with DEP, a reagent "selective" for His (Miles, 1977; Lundblad & Noyes, 1984), the role of His residues in the activity of PLD from *Streptomyces* PMF, which is one of the two PLDs recently isolated by us (Carrea et al., 1995). The enzyme, which was homogeneous by SDS−PAGE, capillary electrophoresis, and analytical gel filtration, was found to be a monomer with a molecular mass of 53 860 Da, as assessed by MALDI mass spectrometry, and showed an isoelectric point of 9.1 and pH optima between 4 and 6 (Carrea et al., 1995). The results here reported have provided evidence that His is not essential for activity and, more importantly, have demonstrated that DEP inactivates the enzyme by chemical modification of Lys residues. The involvement of Lys in PLD activity has been confirmed by studies with PLP (Rippa et al., 1967; Hartman & Norton, 1977) which have shown that enzyme activity is lost after modification of one Lys residue.

MATERIALS AND METHODS

Materials. PLD from *Streptomyces* PMF (42 units/mg) was purified to homogeneity as previously described (Carrea et al., 1995). The enzyme concentration was determined by amino acid analysis after hydrochloric hydrolysis (Carrea et al., 1995). DEP, PLP, hydroxylamine hydrochloride, and sodium borohydride were obtained from Aldrich and DCPC was from Sigma. All other reagents and compounds were of analytical grade.

Enzyme Assay. PLD activity was determined spectrophotometrically in 0.05 M Tris buffer, pH 8, by monitoring the hydrolysis of phosphatidyl *p*-nitrophenol to phosphatidic acid and *p*-nitrophenol at 405 nm (D'Arrigo et al., 1995).

Inactivation of PLD by DEP and Spectral Measurement. In a typical experiment, the enzyme (0.4 mg/mL, 7 μM) was incubated with varying concentrations of DEP in 0.075 M potassium phosphate buffer, pH 6.5, at 25 °C. The DEP solution was freshly prepared by diluting the reagent in ice-cold absolute ethanol. The time course of inactivation was followed by measuring the residual enzyme activity in aliquots removed at different times. The activity was expressed as a percentage of the activity of the enzyme incubated similarly but without DEP. Inactivation was studied in the range of pH 5.3−8.3.

The time course of N-carbethoxylation of His residues of PLD (0.4 mg/mL) was determined by recording continuously the change in absorbance at 240 nm. The stoichiometry of the formation of modified His residues was calculated from the difference in absorbance between treated and untreated enzyme at the same concentration (ϵ_{240} = 3200 M^{-1} cm^{-1}; Miles, 1977). N-Carbethoxylation of PLD was investigated in the range of pH 6.1−8.3.

Hydroxylamine Treatment. The DEP-inactivated enzyme was incubated at 25 °C with 0.1 M hydroxylamine at pH 6.5−8. Untreated enzyme was incubated similarly. The time course of reactivation was followed by measuring the enzyme activity in aliquots removed at different times. The time of restoring His residues from the *N*-carbethoxyhistidine form was followed by recording continuously the change in absorbance at 240 nm.

Dinitrophenylation. PLD samples (0.4 mg/mL) were treated with DEP under different conditions. At set times, 6 mM histidine was added to block unreacted DEP and the samples were dialyzed in the cold against distilled water and then lyophilized. The samples were dissolved (0.6 mg/mL) in a 2% aqueous solution of triethylamine containing 190 mM FDNB and reacted for 3 h at 25 °C in the dark. The samples were then lyophilized, extracted 5 times with ethyl acetate to remove unreacted FDNB, dried under vacuum, and hydrolyzed with 6 M HCl for 18 h at 110 °C before amino acid analysis.

Inactivation of PLD by PLP and Spectral Measurement. The enzyme (0.7 mg/mL) was incubated in 2 mM PLP in 0.2 M sodium carbonate buffer, pH 8.5, 15 °C. At various times aliquots (1 mL) were drawn and a drop of octyl alcohol added to prevent foaming. A freshly prepared solution of sodium borohydride (0.6 M) in 1 mM sodium hydroxide (final concentration 45 mM) was finally added. After reduction, the various aliquots were tested for remaining enzymatic activity and dialyzed exhaustively against 50 mM sodium carbonate, pH 8. The ratios of moles of PLP bound per mole of enzyme were calculated with an ϵ_{325} = 10 000 M^{-1} cm^{-1} (Forrey et al., 1971) for N^6-phosphopyridoxyllysine and an ϵ_{280} = 108 000 M^{-1} cm^{-1} for the enzyme.

Determination of Sulfhydryl Residues. The sulfhydryl content of PLD was assayed with DTNB (Ellman, 1959) in 0.1 M sodium phosphate buffer, pH 8, and 6 M guanidine hydrochloride.

Spectroscopic Studies. Circular dichroism measurements of treated and untreated PLD were made with a Jasco 500 A spectropolarimeter at 25 °C. The intrinsic protein fluorescence of the enzyme, excited at 280 nm, was measured in a Jasco FP-550 spectrofluorometer at 25 °C.

Analytical Gel Filtration. Gel filtration of treated and untreated PLD was carried out on a Superdex 75 HR 10/30 column (Pharmacia) connected with a Jasco HPLC apparatus and equilibrated with 20 mM sodium phosphate buffer, pH 7, containing 180 mM sodium chloride. The flow rate was 0.4 mL/min and reading was at 280 nm.

Effect of Substrate on PLD Inactivation. The inactivation of PLD by 1 mM DEP was studied in the presence of 2−15 mM DCPC in 0.075 M potassium phosphate buffer, pH 7, 25 °C. The effect of DCPC (4−16 mM) on PLD inactivation by 2 mM PLP, followed by sodium borohydride reduction, was studied in 0.2 M sodium carbonate buffer, pH 8.5, 15 °C.

RESULTS

Reaction of PLD with DEP. The inactivation of PLD with DEP followed pseudo-first-order kinetics. The loss of activity over time, corrected for hydrolysis of DEP in buffer, is described by eq 1, in which A/A_0 is the fraction of activity

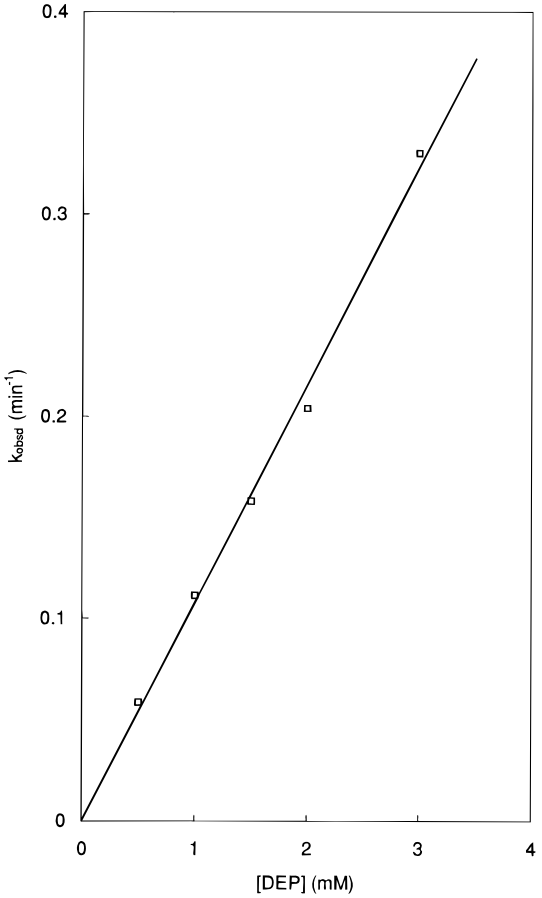

FIGURE 1: Plot of apparent first-order rate constants for PLD inactivation (k_{obsd}) at various concentrations of DEP against concentrations of the reagent.

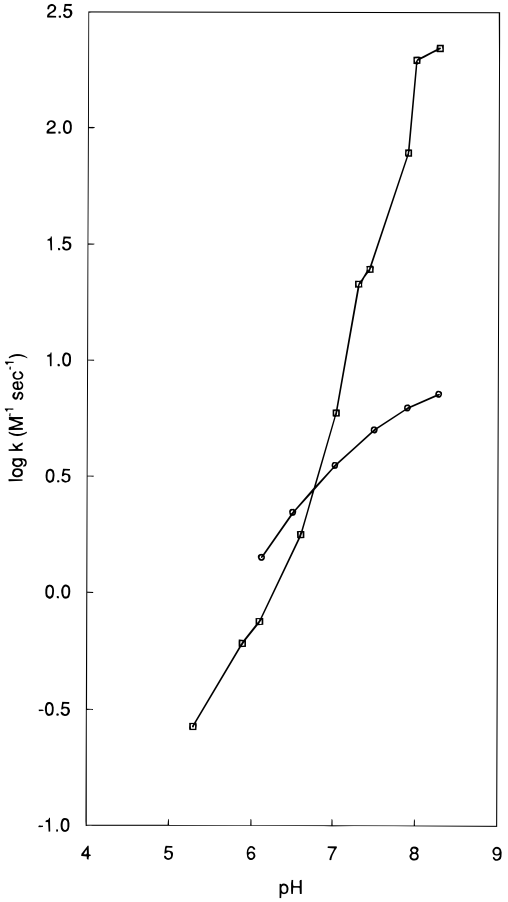

FIGURE 2: Effect of pH on the logarithm of second-order rate constants of inactivation of PLD (0.4 mg/mL) by DEP (□) and the logarithm of the second-order rate constants for reaction of DEP with the His residues of PLD (0.4 mg/mL) (○). The second-order rate constants of enzyme inactivation and carbethoxylation were calculated by taking into account the first-order rate constants for the hydrolysis of DEP at the different pHs (k' in eq 1). For details see text.

remaining at time t, I_0 is the initial concentration of DEP, k is the bimolecular rate constant for the loss of enzyme activity, and k' is the first-order rate constant for the hydrolysis of DEP by buffer (Gomi & Fujioka, 1983). The value of k' at 25 °C in 0.075 M potassium phosphate buffer, pH 6.5, was determined to be 0.59×10^{-3} s^{-1}.

$$\ln(A/A_0) = -(k/k')I_0(1 - e^{-k't}) \quad (1)$$

The data obtained from inactivation experiments of the enzyme at various concentrations of DEP gave straight lines in a plot of the logarithm of remaining activity against $(1 - e^{-k't})/k'$ (not shown). A plot of pseudo-first-order rate constants for inactivation (k_{obsd}) against DEP concentration was linear (Figure 1), and from linear regression analysis of the data, the second-order rate constant for inactivation (k) at pH 6.5 and 25 °C was calculated to be 1.8 M^{-1} s^{-1}.

The pH dependence of the inactivation of PLD by DEP was studied over the range of pH 5.3−8.3. The plot of the logarithms of the second-order rate constants for inactivation (k) against pH is shown in Figure 2, with the plot of the logarithms of the second-order rate constants for reaction of DEP with the His residues of PLD, as determined by continuously recording the change in absorbance at 240 nm. The rate of inactivation of PLD as a function of pH differed markedly from the rate of reaction of the His residues of the enzyme, and indeed, at pH 8.3 the inactivation rate (222 M^{-1} s^{-1}) was about 30 times higher than the rate of His modification (7.2 M^{-1} s^{-1}), while at pH 6.1 the inactivation rate (0.7 M^{-1} s^{-1}) was half the value of the rate of His modification (1.4 M^{-1} s^{-1}). It should be emphasized that 5 His residues per enzyme molecule were carbethoxylated at the various pHs when PLD was completely inactivated by DEP and that all of them apparently reacted at the same rate as inferred by the finding that the time course of carbethoxylation was monophasic. In fact, straight lines were obtained when plotting the logarithm of Δ absorbance at 240 nm against $(1 - e^{-k't})/k'$. According to Wijnands and Müller (1982), Δ absorbance was normalized by giving the difference of absorbance at zero time of reaction with DEP a value of 1, and when enzyme inactivation was complete (stable absorbance corresponding to 5 His residues modified per enzyme molecule) a value of 0. The profound discrepancy between the rates of enzyme inactivation and His carbethoxylation strongly suggests that the modification of His residues is not responsible for the loss of enzyme activity.

Hydroxylamine Treatment. Incubation of the enzyme inactivated by DEP at different pHs (5.3−8.3) with 0.1 M hydroxylamine in the pH range 6.5−8 for up to 3 h did not significantly regenerate the activity of PLD, although the reversal of modification of His residues was almost complete (92%, average of 6 experiments). These results support the view that the His residues modified by DEP are not essential for activity. It should be noted that hydroxylamine treatment affected neither the activity nor the conformation of PLD.

Table 1: Residual Lys Residues after FDNB Treatment of DEP-Inactivated Enzyme[a]

sample	% inactivation by DEP	residual Lys residues after FDNP treatment[b]
control	0	1.1
enzyme inactivated with DEP at pH 6.5[c]	98	18.2
enzyme inactivated with DEP at pH 8.3[d]	99	22.1

[a] For details see Materials and Methods. [b] Expressed as moles per mole of enzyme. Data are means for two determinations. [c] PLD was incubated at 25 °C with 3 mM DEP in 0.075 M potassium phosphate buffer, pH 6.5, for 30 min. [d] PLD was incubated at 25 °C with 2 mM DEP in 0.075 M potassium phosphate buffer, pH 8.3, for 2 min.

Amino Acid Residues Other Than His Modified by DEP. The DTNB assay showed that no sulfhydryl group is present in PLD. This rules out the possibility that enzyme inactivation can be caused by DEP modification of a Cys residue. The modification of Tyr residues can be excluded because there was no decrease in absorbance at 278 nm, ascribable to O-carbethoxylation of Tyr residues (Miles, 1977), when PLD was treated with DEP under conditions producing complete enzyme inactivation. The hypothesis of involvement of Ser residues should also be rejected because it has been reported for α-chymotrypsin that the rate of enzyme reactivation and concomitant hydrolysis of the carbethoxylated essential Ser by hydroxylamine are very high (Melchior & Fahrney, 1970).

To find out whether Lys residues react with DEP, PLD was dinitrophenylated. The experiments were based on the ability of the carbethoxyl group to protect against reaction with FDNB (Melchior & Fahrney, 1970; Wells, 1973; Pasta et al., 1987). Table 1 shows that DEP protected, after correction for control, 17 Lys out of 31 present per enzyme molecule (Carrea et al., 1995) at pH 6.5 or 21 Lys at pH 8.3 respectively, against dinitrophenylation. Such high values of Lys residues modified by DEP are quite surprising even though other cases of Lys modified by DEP have been described previously (Wells, 1973; Burstein et al., 1974; Pasta et al., 1987). The basic character of PLD (isoelectric point 9.1; Carrea et al., 1995) could partly explain this behavior. These results make it difficult to understand whether enzyme inactivation is caused by reaction of DEP with an essential Lys residue or by enzyme conformational variations caused by such extensive modification of Lys residues. However, it should be emphasized that the inactivated enzyme did not show appreciable differences from the native enzyme in intrinsic protein fluorescence, molar ellipticity (circular dichroism analyses), or hydrodynamic volume as measured by gel filtration, and this indicates that DEP treatment did not induce conformational modifications or aggregation of the protein.

Reaction of PLD with PLP. Treatment of PLD with PLP followed by sodium borohydride reduction caused irreversible inactivation of the enzyme according to pseudo-first-order kinetics (Figure 3). The second-order rate constant for inactivation at pH 8.5 and 15 °C was calculated to be 3.5 M^{-1} s^{-1}.

The remaining PLD activity (*a*) was linearly related to the extent of Lys residue modification (*m*) and, on extrapolation of the plot to 0% residual activity, correlated with modification of 4 Lys residues per enzyme molecule (Figure 4). The problem of how to differentiate essential from nonessential residues was addressed by the method of Tsou (1962), which uses the relationship described by eq 2, in which *a* is the remaining activity when *m* groups have reacted, *p* is the total number of groups modified, and *i* is the number of critical residue(s) for catalytic activity. The

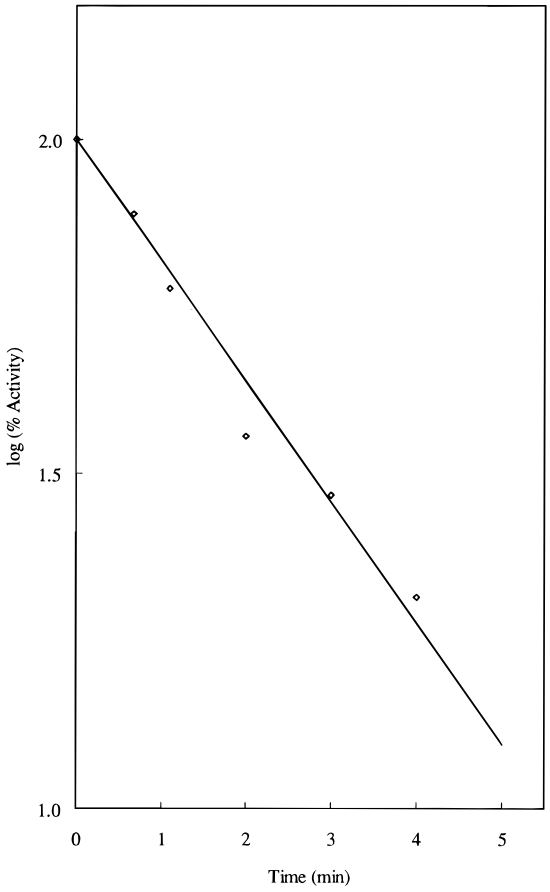

FIGURE 3: Inactivation of PLD by PLP. The enzyme (0.7 mg/mL) was incubated with 2 mM PLP in 0.2 M sodium carbonate buffer, pH 8.5, 15 °C, and then reduced with 45 mM sodium borohydride.

$$a^{1/i} = (p - m)/p \qquad (2)$$

number of essential residue(s) is that value of *i*, for a given value of *p*, that gives a straight line when $a^{1/i}$ is plotted against *m*. A straight line shows a satisfactory fit to the observed experimental data for the inactivation of PLD with PLP when $p = 4$ and $i = 1$ (Figure 4). The other lines shown in Figure 4 are theoretical curves fitted to the same data, also for $p = 4$, but assuming that more than 1 critical residue ($i > 1$) is responsible for the inactivation.

Effect of Substrate on PLD Inactivation. To study the effect of substrate on PLD inactivation, we used, as already done by Wells (1973) for phospholipase A_2 inactivation by DEP, the water soluble DCPC. Table 2 shows that the substrate protected in a concentration-dependent manner the enzyme against inactivation by both DEP and PLP. At the highest DCPC concentrations the second-order rate constants for inactivation were 6−7 times lower than those obtained in the absence of substrate.

DISCUSSION

The treatment of PLD with DEP modified 5 His residues per enzyme molecule, but none of these was essential for

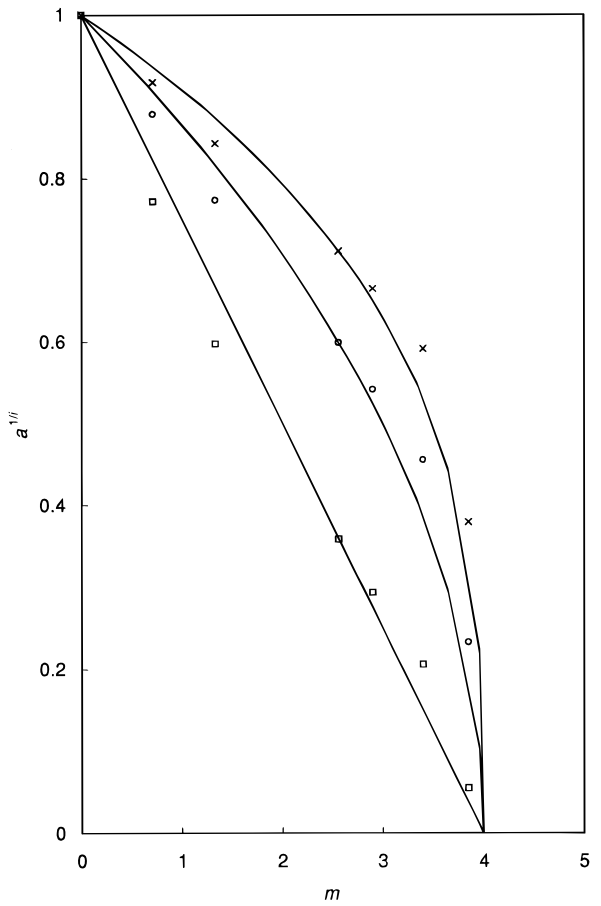

FIGURE 4: Relationship between remaining activity (a) and the number of Lys residues modified by PLP per enzyme molecule (m). Lines were obtained from eq 2, with $p = 4$ and $i = 1$(□), 2 (○), and 3 (×).

Table 2: Effect of DCPC on PLD Inactivation by DEP or PLP[a]

conditions	second-order rate const for inactn (M^{-1} s^{-1})	conditions	second-order rate const for inactn (M^{-1} s^{-1})
DEP	5.8	PLP	3.5
DEP + 2 mM DCPC	3.6	PLP + 4 mM DCPC	2.2
DEP + 4 mM DCPC	3.1	PLP + 13 mM DCPC	1.1
DEP + 8 mM DCPC	1.4	PLP + 16 mM DCPC	0.5
DEP + 15 mM DCPC	1.0		

[a] For conditions, see Materials and Methods.

activity. This statement is based on: (a) the profound discrepancy between the rate of enzyme inactivation and the rate of modification of His residues in PLD as a function of pH (Figure 2) and (b) the finding that the reversal of His modification by hydroxylamine treatment was not accompanied by recovery of activity. It could also be hypothesized that an essential His residue was masked by the irreversible modification of another essential residue such as Lys, but this possibility seems not to be well grounded. In fact, if at pH values above the crossing point between the curve of enzyme inactivation and that of His modification, the higher reactivity of an essential Lys could indeed prevent us from recognizing the existence of a slower-reacting essential His, this could not happen at pH values below the crossing point where the opposite was true, i.e., the rates of His modification were higher than those of PLD inactivation (Figure 2). Therefore, if an essential His was present, the rates of PLD inactivation at low pHs had to be higher and coincident with those of His modification.

The lack of an essential His in PLD from *Streptomyces* PMF was somewhat unexpected because previous studies based on the chemical modification by DEP of PLD from cabbage (Lee et al., 1989) or on formic acid-induced mutagenesis of PLD from C. *pseudotuberculosis* (Haynes et al., 1992) had indicated that there is an essential His residue in these enzymes. It should be noted, however, that, in the first case (Lee et al., 1989), no study of reversal by hydroxylamine of enzyme inactivation and of the correlation between inactivation and His modification at various pHs was carried out to unambiguously demonstrate the involvement of His in enzyme activity.

DEP also modified up to 20 Lys residues per enzyme molecule, as demonstrated by dinitrophenylation experiments (Table 1). The fact that Tyr, Ser, and Cys (not present in PLD) residues, and the conformation and the molecular size of the enzyme, were not modified suggests that there is a Lys residue essential for PLD activity. The modification by DEP of Lys residues important for activity has been described previously for phospholipase A_2 from *Crotalus adamanteus* (Wells, 1973), 3α,20β-hydroxysteroid dehydrogenase (Pasta et al., 1987), phenol hydroxylase (Sejlitz & Neujahr, 1987), glutamate dehydrogenase from *Chlorella pyrenoidosa* (Shalitov et al., 1989), and glutathione *S*-transferase CL 3-3 (Chang & Tam, 1993).

The involvement of a Lys residue in PLD activity was confirmed by experiments with PLP which inactivated the enzyme with concomitant modification of 4 Lys residues, only one of which was found to be essential by analysis of the results with Tsou's kinetic method (Figure 4). The remarkable protection by substrate of PLD inactivation by DEP and PLP strongly suggests that the essential Lys residue is located in or near the substrate binding site of the enzyme. Experiments with PLP have shown the presence of Lys residues essential for activity also in another phospholipase, namely, phospholipase C from *Bacillus cereus* (Aurebekk & Little, 1977).

Concerning the role of Lys in PLD catalysis, it could be imagined that in its protonated form the residue acts as a counterion for the phosphodiester group of phospholipids, thus favoring substrate binding. Support to this hypothesis comes from the analysis of the pH profile for PLD activity which was found to be bell-shaped and with approximate pK_a values of 3.8 and 8 for the ascending and descending legs (Carrea et al., 1995). Therefore, the decreases of activity at higher pHs would reflect the progressive dissociation of protonated Lys which renders the residues unable to bind the substrate. Instead, the decreases of activity at lower pHs suggest the involvement in PLD catalysis of other residues with lower pK_a values. However, these hypotheses should be considered with some caution because: (1) the extremely fast reaction rates at pHs higher than 8.3 prevented the exact determination of the pK_a of PLD inactivation, and (2) the pK_a values obtained by activity measurements can differ from those obtained by inactivation experiments since in the first case the binding of substrates perturbs the pK_a of the active site (Fersht, 1985).

The enzyme partially inactivated with DEP or PLP (25% and 6% residual activity) had a K_m value for the substrate identical with that of the native enzyme, and this rules out

the possibility that the loss of activity after modification might also be due to an increase in K_m values.

In conclusion, the study has demonstrated through the combined use of DEP and PLP that a Lys residue is essential for the activity of PLD from *Streptomyces* PMF. Contrary to serine hydrolases, which phospholipases D resemble since they presumably act through the formation of a phosphatidyl-enzyme intermediate (Bruzick & Tsai, 1984), PLD from *Streptomyces* PMF does not contain any essential His residue. The results also show that DEP is far from being a reagent selective for His, because for PLD up to 20 Lys residues per enzyme molecule were rapidly modified by this reagent.

REFERENCES

Aurebekk, D., & Little, C. (1977) *Biochem. J. 161*, 159−165.

Bruzik, K., & Tsai, M. D. (1984) *Biochemistry 23*, 1656−1661.

Burstein, Y., Walsh, K. A., & Neurath, H. (1974) *Biochemistry 13*, 205−210.

Carrea, G., D'Arrigo, P., Piergianni, V., Roncaglio, S., Secundo F., & Servi, S. (1995) *Biochim. Biophys. Acta 1255*, 273−279.

Chang, L.-H., & Tam, M. F. (1993) *Eur. J. Biochem. 211*, 805−811.

D'Arrigo, P., de Ferra, L., Piergianni, V., Ricci, A., Scarcelli, D., & Servi, S. (1994) *J. Chem. Soc., Chem. Commun.*, 1709−1710.

D'Arrigo, P., Piergianni, V., Ricci, A., Scarcelli, D., & Servi, S. (1995) *Anal. Chim. Acta 304*, 249−254.

Dennis, E. A., Ed. (1991) *Phospholipases, Methods in Enzymology* Vol. 197, Academic, San Diego.

Dennis, E. A. (1994) *J. Biol. Chem. 269*, 13057−13060.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, pp 155−175, Freeman and Co., New York.

Forrey, A. W., Olsgaard, R. B., Nolan, C., & Fischer, E. H. (1971) *Biochimie 53*, 269−281.

Gomi, T., & Fuijoka, M. (1983) *Biochemistry 22*, 137−143.

Hartman, F., & Norton, I. L. (1977) *Methods Enzymol. 47*, 479−498.

Haynes, J. A., Tkalcevic, J., & Nisbet, J. T. (1992) *Gene 119*, 119−121.

Juneja, L. R., Hibi, H., Inagaki, N., Yamane, T., & Shimizu, S. (1987) *Enzyme Microb. Technol. 9*, 350−354.

Juneja, L. R., Kazuoka, T., Yamane, T., & Shimizu, S. (1988) *Biochim. Biophys. Acta 960*, 334−341.

Lee, H., Choi, M.-U., & Koh, E.-H., (1989) *Korean Biochem. J. 22*, 487−493.

Lundblad, R. L., & Noyes, C. M. (1984) in *Chemical Reagents for Protein Modification*, Vol. 1, pp 105−125, CRC Press, West Palm Beach, FL.

Melchior, W. B., & Fahrney, D. (1970) *Biochemistry 9*, 251−258.

Miles, E. W. (1977) *Methods Enzymol. 47*, 431−442.

Pasta, P., Mazzola, G., & Carrea, G. (1987) *Biochemistry 26*, 1247−1251.

Rippa, M., Spanio, L., & Pontremoli, S. (1967) *Arch. Biochem. Biophys. 118*, 48−57.

Sejlitz, T., & Neujahr, H. Y. (1987) *Eur. J. Biochem. 170*, 351−356.

Shalitov, V. R., Loseva, L. P., Bendianishvili, M. V., Shubin, V. V., & Kretovich, W. L. (1989) *Biochim. Biophys. Acta 994*, 222−228.

Tsou, C.-L. (1962) *Sci. Sin. (Engl. Ed.) 11*, 1535−1558.

Van Nieuwenhuyzen, W. (1981) *J. Am. Oil Chem. Soc. 58*, 886−888.

Wells, M. A. (1973) *Biochemistry 12*, 1086−1093.

Wijnands, R. A., & Müller, F. (1982) *Biochemistry 21*, 6639−6646.

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