A Shift in the Optimum pH of Phospholipase D Produced by Activating Long-Chain Anions

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1. The activity of phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) towards ultrasonically treated phosphatidylcholine or large phosphatidylcholine particles activated with ether was maximal near pH 5, and there was little activity above pH 6. 2. When the enzyme was activated by the addition of phosphatidic acid to large phosphatidylcholine particles the pH optimum was shifted to pH 6.5 irrespective of the amount of activator added. 3. When the enzyme was activated with low concentrations of dodecyl sulphate the pH optimum was 5.5 with little activity above pH 6. With higher concentrations of dodecyl sulphate the pH-activity profile was shifted upwards towards a pH optimum of 6.5-6.6, the magnitude of the shift depending on the extent of the hydrolysis. 4. The shifts in the pH-activity profiles cannot be correlated with changes in the 'surface pH' of the substrate particles calculated from the measurement of their ζ -potentials (electrophoretic mobilities).

Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) is widely distributed in plant tissues, and it can cause a major breakdown of the phospholipids of cell organelles, e.g. mitochondria, during the isolation of these from plant tissues (J. Eichberg & R. M. C. Dawson, unpublished work). A range of optimum pH values for phospholipase D has been reported, varying from 4.8 for the enzyme in spinach leaves and sugar beet (Kates, 1954) to 6.0 in rubber latex (Smith, 1954). However, for the particulate or the soluble enzyme prepared from one of its richest known sources, the *Brassica* genus, the pH optimum measured with diethyl ether as an activator has consistently been reported to be about 5.5 (Kates, 1954; Davidson & Long, 1958; Dawson & Hemington, 1967).

In this paper evidence is presented that the optimum pH for the soluble cabbage enzyme is about 4.9 when it is hydrolysing ultrasonically treated phosphatidylcholine particles and 5.2 when it is activated with ether and hydrolysing large phosphatidylcholine particles, but that the optimum is shifted as much as 1.5 pH units higher when anionic amphipathic substances are used to activate the enzymic reaction (Dawson & Hemington, 1967). Some of the possible reasons for this shift have been investigated and the results are discussed in terms of changes in the H^+ concentration in the surface phase adjacent to the substrate.

METHODS

Materials and phospholipase D assay. The soluble enzyme from the heart of Savoy cabbage was purified by the procedure of Davidson & Long (1958) as far as the acetone precipitation stage. The total activity obtained and the activity/mg. of protein varied considerably with each preparation and are probably dependent on factors such as the maturity of the cabbage. Enzymic activity was measured substantially by the procedures of Dawson & Hemington (1967).

Phosphatidylcholine (ovolecithin) was prepared as described by Dawson (1963). The lipid-solvent-soluble anionic activators (stearyl sulphate, phosphatidic acid, dicetylphosphoric acid, monocetylphosphoric acid and triphosphoinositide) were mixed with the phosphatidylcholine (5 μ moles/assay tube) in solution in organic solvents and evaporated to dryness, and the lipid mixtures were suspended in water. Sodium dodecyl sulphate was added to the phosphatidylcholine suspension as a concentrated aqueous solution; $CaCl_2$ and buffer were then added to the lipid suspension so that the final concentrations in the incubation mixture were 40 mM- $CaCl_2$ and 50 mM-buffer. For most experiments, sodium acetate-acetic acid buffers were used below pH 6.0, and $\beta\beta'$ -dimethylglutaric acid-NaOH buffers were used at pH 6.0 and above. In a few experiments $\beta\beta'$ -dimethylglutaric acid-NaOH buffers were used throughout the pH range. The final pH of the incubation mixture was measured with a glass electrode, and the enzyme was added to give a total volume of 1.2 ml. Incubation was carried out at 27°, and the choline liberated was assayed in the deproteinized supernatant by an iodine precipitation method (Dawson & Hemington, 1967).

Electrophoretic mobilities. The mobilities of the substrate particles were measured at 27° in a horizontal electrophoresis cell (Bangham, Flemans, Heard & Seaman, 1958). To observe the lipid particles clearly the suspensions had to be diluted fourfold compared with their final concentration in the enzymic assay, but care was taken to maintain the ionic environment of the particles constant.

RESULTS

Initial experiments clearly showed that the pH of the complete incubation medium before the addition of the enzyme was a few tenths of a pH unit lower than that of the buffer used. This effect was almost entirely due to the high concentration of calcium chloride, which was necessary to stimulate the enzymic reaction maximally. Even though the pH of the added calcium chloride (6.6) was higher than that of the buffer, it still caused a decrease in the pH of the system, presumably by reacting with dissociated acetate or $\beta\beta'$ -dimethylglutarate ions in the buffer, and thus causing a proton release by further dissociation of the weak acid. All pH values reported are therefore the measured values rather than the values of the added buffers.

pH optima with substrates of ultrasonically treated and ether-activated phosphatidylcholine. An ultrasonically treated phosphatidylcholine preparation gave a sharp pH optimum at 4.9 with a very marked fall of activity at higher pH values, so that at pH 5.5 only low activity could be detected (Fig. 1a). The use of larger phosphatidylcholine particles and activation of the system with diethyl ether (Dawson & Hemington, 1967) gave a slightly higher pH optimum and the activity fell much less sharply as the pH was increased (Fig. 1a). The optimum pH value was slightly below those previously reported with ether activation (Kates, 1954; Davidson & Long, 1958; Dawson & Hemington, 1967), probably because of the allowance made in the present investigation for the effect of Ca^{2+} on the pH of the buffer.

The sharp fall in activity observed above pH 5 with the ultrasonically treated phosphatidylcholine was not due to an irreversible denaturation of the enzyme at the higher pH values in the presence of the highly dispersed phosphatidylcholine substrate. The enzyme could be preincubated with the ultrasonically treated phosphatidylcholine- Ca^{2+} mixture at pH values above 5.5 without losing its activity as subsequently measured in a different assay system. On the other hand, the marked fall in activity above pH 5 was largely prevented by increasing the concentration of calcium chloride in the system from 40mM to 0.4M, although the pH optimum was not increased significantly (Fig. 2).

pH optima with phosphatidylcholine substrate and

Fig. 1. pH-activity curves for cabbage phospholipase D. Incubation was carried out as described in the Methods section. Sodium acetate-acetic acid buffers (50mM) were used below pH 6.0 and $\beta\beta'$ -dimethylglutaric acid-NaOH buffers (50mM) above pH 6.0. All the systems contained 5 μ moles of phosphatidylcholine. The same enzyme preparation was used for all the experiments. (a) ●, 15% (v/v) diethyl ether added, 0.02 ml. of enzyme used (choline liberated has been multiplied by 10); ○, phosphatidylcholine ultrasonically treated for 15 min. at 0° (MSE 60w instrument), 0.02 ml. of enzyme used. (b) ▲, 0.5 μ mole of dodecyl sulphate added, 0.02 ml. of enzyme used; △, 2.5 μ moles of dodecyl sulphate added, 5 μ l. of enzyme used. (c) ■, 0.5 μ mole of phosphatidic acid added, 0.02 ml. of enzyme used; □, 1.5 μ moles of phosphatidic acid added, 5 μ l. of enzyme used.

anionic amphipathic activators. In agreement with the results of Dawson & Hemington (1967), the enzymic activity was found to be much greater in systems in which certain anionic amphipathic substances were mixed with the phosphatidylcholine than in systems with ether as an activator or with ultrasonically treated phosphatidylcholine

Fig. 2. Effect of calcium chloride concentration on the pH-activity profile of cabbage phospholipase D acting on a ultrasonically treated phosphatidylcholine substrate. The ultrasonically treated phosphatidylcholine (5 μ moles) was suspended in 1.2 ml. of incubation medium containing $\beta\beta'$ -dimethylglutaric acid-NaOH buffer (50 mM) and 40 mM-CaCl₂ (○) or 0.40 M-CaCl₂ (●). The pH was measured with a glass electrode and the mixture was incubated for 10 min. at 27° with 0.1 ml. of enzyme containing 0.66 mg. of protein.

Fig. 3. Variation of the pH optimum of hydrolysis by cabbage phospholipase D of phosphatidylcholine + dodecyl sulphate particles. The incubation media contained 5 μ moles of phosphatidylcholine in 1.2 ml. of 40 mM-CaCl₂-50 mM- $\beta\beta'$ -dimethylglutaric acid-NaOH buffer at the appropriate pH. ○, 2.5 μ moles of dodecyl sulphate added, incubated for 6 min., 0.9 μ g. of enzyme protein used; ●, 2.5 μ moles of dodecyl sulphate added, incubated for 24 min., 0.9 μ g. of enzyme protein used; △, 0.5 μ mole of dodecyl sulphate added, incubated for 24 min., 5.1 μ g. of enzyme protein used.

as a substrate (Fig. 1). Further, in the presence of phosphatidic acid the pH optimum was shifted about 1.5 units higher, to pH 6.5 (Fig. 1c). The higher optimum was found both when the enzyme was activated maximally with 1.5 μ moles of phosphatidic acid/5 μ moles of phosphatidylcholine and when it was treated with a suboptimum amount of phosphatidic acid (0.5 μ mole/5 μ moles). The higher pH optimum occurred with all amounts of phosphatidic acid tested that were sufficient to activate the reaction.

Initial experiments indicated that a similar upward shift of the pH optimum was produced when the enzyme was activated maximally with 2.5 μ moles of dodecyl sulphate/5 μ moles of phosphatidylcholine (Fig. 1b). However, a decrease in the concentration of dodecyl sulphate resulted, not only in a decreased activity, but also in a smaller shift of the pH optimum from that of phosphatidylcholine alone (Fig. 1b). Similar results were obtained with stearyl sulphate. Further experiments with the optimum amount of dodecyl sulphate (2.5 μ moles/5 μ moles) showed that, although the pH-activity curve was always extended to show considerable activity between pH 6 and 7, the actual optimum depended on the amount of hydrolysis that occurred. Fig. 3 shows that, as the time of incubation was increased from 6 to 24 min. and the percentage hydrolysis in-

creased, the pH-activity profile changed from a broad flat optimum between pH 5.5 and 6.5 to a clearly defined optimum at pH 6.5.

In some experiments in which the percentage hydrolysis was very low, the optimum was as low as pH 5.5, although the peak was always broad with considerable activity as high as pH 7. On the other hand, with a small amount of dodecyl sulphate (0.5 μ mole/5 μ moles of phosphatidylcholine) as activator and a higher enzyme concentration, the optimum was about pH 5.3 with little activity above pH 6, even though the percentage hydrolysis of substrate was greater in this instance than in the system containing concentrations of dodecyl sulphate that produced maximal activation with smaller amounts of enzyme (Fig. 3).

In a number of experiments, the time-course of the enzymic hydrolysis was studied at pH 5.4 and 6.4 with different enzyme concentrations and with optimum amounts of phosphatidic acid and dodecyl sulphate as activators. Although at both pH values the rate of choline liberation fell somewhat as the reaction proceeded, the results did not suggest that the shift of the pH-activity profile to higher pH values was an artifact produced by a greater instability of the enzyme at lower pH values in the presence of the activators.

Table 1. *Comparison of the electrophoretic mobilities of substrate particles and the pH optimum of their hydrolysis by cabbage phospholipase D*

The electrophoretic mobilities were measured at the optimum pH with either acetate or $\beta\beta'$ -dimethylglutarate buffers of the same ionic strength. The substrate ratios given are molar ratios.

Substrate	Electrophoretic mobility ($\mu\text{m./sec./v/cm.}$)		Optimum pH
	Ca ²⁺ (0.04 M) present	Ca ²⁺ omitted	
Phosphatidylcholine	+2.1	0	4.9 (ultrasonically treated) 5.2 (with ether)
Phosphatidylcholine-dodecyl sulphate (5:1)	+0.1	-11.5	5.4
Phosphatidylcholine-dodecyl sulphate (2:1)	-0.9	-13.8	5.6-6.8 (depending on amount of hydrolysis)
Phosphatidylcholine-phosphatidic acid (10:1)	+1.5	-4.5	6.5
Phosphatidylcholine-phosphatidic acid (10:3)	+1.3	-11.5	6.6

The shift of the pH optimum to higher values in the presence of an optimum concentration of dodecyl sulphate was observed even when the reaction was carried out in the presence of 1M-sodium chloride. When the phosphatidylcholine substrate was both ultrasonically treated and activated with optimum dodecyl sulphate concentration the activity was considerably higher than with either treatment alone, and the pH optimum was 6.6.

The anionic amphipathic substances produced a similar upward shift in the pH-activity profile both when acetate buffers were used below pH 6.0 and $\beta\beta'$ -dimethylglutarate buffers at pH 6.0 and above, and when $\beta\beta'$ -dimethylglutarate buffers were used throughout the pH range. The same upward shift in the pH profile was observed when the buffer was eliminated and the production of phosphatidic acid was continuously monitored by titration with a pH-stat (Radiometer, Copenhagen, Denmark). The release of 1 μmole of choline by the enzyme required the expected addition of 1 μmole of sodium hydroxide to maintain the pH constant at the selected point. There was a slight fall in the rate as the hydrolysis proceeded, but the initial rates again showed a pronounced change to a higher pH-activity profile in the presence of the anionic amphipathic activators.

pH optima and the electrophoretic mobilities of substrate particles. Because ionic amphipathic substances cause a change in the electrostatic field at a lipid/water interface and thus affect the concentration of protons in the surface phase, the pH optima for the enzymic hydrolysis with dodecyl sulphate and phosphatidic acid as activators were compared with the electrophoretic mobilities of the substrate particles. Both in the complete system containing Ca²⁺ and in the absence of Ca²⁺ there was no evidence of a correlation between the initial electrophoretic mobilities of the substrate particles

and the pH optima (Table 1). The liberation of phosphatidic acid during the reaction is unlikely to alter the ζ -potential of the substrate significantly, owing to the Ca²⁺ present (Dawson & Hemington, 1967).

DISCUSSION

The function of phospholipase D in higher plants is not yet apparent. If the enzyme shows the same pH range for hydrolysis *in vivo* as it does towards ultrasonically treated phosphatidylcholine, i.e. little activity above pH 5.5, it is unlikely to be active under normal physiological conditions. It is clear that in the presence of the lipoidal hydrolysis product of the enzyme, phosphatidic acid, not only is the hydrolysis greatly stimulated, but activity is observed at pH values nearer to the physiological, i.e. above pH 6. Presumably activity against ultrasonically treated phosphatidylcholine is not observed above pH 6 because sufficient phosphatidic acid is never liberated to make the reaction autocatalytic. Why phosphatidic acid is so effective in inducing activity above pH 6 and thus shifting the pH optimum cannot be deduced from the present observations. The shift appears to be independent of the amount of phosphatidic acid added or the extent or time of the incubation. It is not a peculiar property of the primary phosphate group, since in further experiments (R. H. Quarles & R. M. C. Dawson, unpublished work) the activator monocetylphosphoric acid (Dawson & Hemington, 1967) gave a pH optimum of about 5.3.

The situation with dodecyl sulphate activation is more complicated. At low concentrations of dodecyl sulphate the optimum pH is at 5.2-5.5 irrespective of how much hydrolysis has occurred. Presumably above pH 6 the reaction is very slow so that little or no phosphatidic acid is formed. With higher concentrations of dodecyl sulphate the reaction can proceed above pH 6, and as phosphatidic acid is

formed the reaction proceeds better at the higher pH values and eventually displays the high pH optimum observed with phosphatidic acid as activator. The shift in the pH-activity profile as the time of incubation is extended is clearly shown in Fig. 3.

One consequence of the addition of anionic amphipathic substances such as phosphatidic acid and dodecyl sulphate to phosphatidylcholine is that the excess of negative groups at the lipid/water interface of the substrate attracts Ca^{2+} and greatly increases the concentration of these ions in the surface phase. Fig. 2 shows that a high concentration of bulk-phase Ca^{2+} does extend the higher range over which the enzyme is operative against an ultrasonically treated phosphatidylcholine substrate. However, the pH optimum is not shifted appreciably towards a higher value, nor does an activation of the hydrolysis occur. Nevertheless, the Ca^{2+} concentration adjacent to the substrate may be elevated much more than tenfold by anionic activators, and this might be partially responsible for the effects observed.

Shifts in the pH optima of enzymes acting at interfaces have often been explained by the electrostatic field at the interface altering the surface pH from that of the bulk buffer phase. For instance, McLaren & Esterman (1957) suggested that the higher pH optimum for chymotrypsin acting on denatured lysozyme adsorbed on kaolinite is due to the latter being negatively charged and attracting H^+ to the interface. Similar explanations have been given for shifts in the pH-activity profiles when trypsin is chemically attached to a co-polymer of maleic acid and ethylene (Goldstein, Levin & Katchalski, 1964) and ficin to carboxymethyl-cellulose (Hornby, Lilly & Crook, 1966). Part of the evidence for such explanations has been the partial reversal of the shift of pH optimum on the addition of high concentrations of salt, which suppresses the negative charge (Goldstein *et al.* 1964). However, in the present system the high Ca^{2+} concentration needed for the enzymic reaction greatly decreases or eliminates the negative ζ -potential of the substrate particles. Further, the high pH optimum is observed even when 1M-sodium chloride is present in addition to the other components of the reaction system.

The pH in the surface phase (S) adjacent to a negatively charged interface differs from that in the bulk phase (B) by the following expression, derived from the Boltzmann equations:

$$\text{pH}_S = \text{pH}_B + (\epsilon\psi/2.3 kT)$$

where k is Boltzmann's constant, T is the absolute temperature, ϵ is the electronic charge and ψ is the potential in the surface phase (Davies & Rideal, 1961). In the present circumstances it is extremely

difficult to know the precise ψ value to be used in such an equation. Measurements of the ζ -potential (electrophoretic mobility) of the substrate particles either in the presence or in the absence of Ca^{2+} show no correlation with the shift in the pH optimum when used in this formula. If therefore the electrostatic field at the interface is responsible for the shift it would have to act at a point nearer to the interface than at the plane of shear between the substrate particle and its fixed counterions, which govern the ζ -potential and consequently the electrophoretic mobility. However, the shift in pH optimum of adipose-tissue lipase produced by ionic surface-active agents is in the opposite direction to that which would be predicted from the asymmetry of proton distribution between surface and bulk phases caused by the amphipathic substance, i.e. cationic detergents produce an increase in the pH optimum and anionic detergents a decrease (Wallach, 1967). Clearly therefore in some instances other mechanisms, e.g. a change in the conformation of the enzyme molecule, must be responsible for the shift in the pH-activity profile.

The results described here showed that the phospholipid/water interface should be added to those examples in the literature in which the pH requirements of an enzyme are altered according to the nature of the surface on which it is acting. It is likely that other enzymes that act on insoluble phospholipid substrates can also have their pH-activity profiles changed according to the conditions at the lipid surface.

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