A Phospholipid-Deacylating System of Bacteria Active in a Frozen Medium

By G. P. HAZLEWOOD and R. M. C. DAWSON

Department of Biochemistry, A.R.C. Institute of Animal Physiology, Babraham,
Cambridge CB2 4AT, U.K.

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1. A phosphatidylcholine-deacylating system present in a *Butyrivibrio* species (probably *fibrisolvens*) shows appreciable activity at low temperatures with a maximum hydrolysis rate at -10°C . 2. The rate at -10°C is higher than at 39°C unless the system at the latter temperature is stimulated by adding oleic acid or sodium dodecyl sulphate. 3. The low-temperature phospholipase activity has an absolute requirement for thiol reagents, e.g. cysteine, dithiothreitol or mercaptoethanol. 4. Ca^{2+} , Mg^{2+} and Mn^{2+} stimulate the activity up to 10mM, but EDTA inhibits; higher concentrations of Ca^{2+} also inhibit. 5. The enhancement of activity at low temperatures appears not to be associated with a crystalline change in the hydrated phospholipid substrate, but depends on the formation of a solid phase in the incubation medium which brings the substrate and bacterial cells into juxtaposition or causes fusion.

We have reported on the properties of a very active phosphoglyceride-deacylating system present in an anaerobic bacterium isolated from rumen fluid and which has been tentatively identified as a strain of *Butyrivibrio fibrisolvens* (Hazlewood & Dawson, 1975). A surprising finding was that the activity of the enzyme system was appreciable even when the substrate-containing medium and bacterial cells were frozen solid at -16°C . The present communication investigates this phenomenon in detail and it is apparent that the formation of a solid phase can markedly activate the rate of enzymic hydrolysis so that under certain incubation conditions the attack at -10°C is more rapid than the hydrolysis at the normal environmental temperature of the bacterium (39°C). Our investigations have shown that the mechanism of the stimulatory effect of freezing is not due to a change in the crystalline structure of the hydrated phosphatidylcholine substrate, but rather the bringing together in close juxtaposition or fusion of the substrate and bacterial cells.

Experimental

Incubation conditions

The isolation of the *Butyrivibrio* sp. and the method of growing shake-flask cultures is described in Hazlewood (1974) and Hazlewood & Dawson (1975). After growing anaerobically to a maximum turbidity the cells were harvested after a further 4–6h incubation when maximum phospholipase activity was apparent (Hazlewood & Dawson, 1975). Cells from each 5ml of culture medium were freshly

harvested by centrifuging at $2000g_{\text{av}}$ and were washed with 0.1M-dimethylglutaric acid/NaOH buffer, pH 6.8, usually containing cysteine (10mM) and Ca^{2+} (1mM) or other additions depending on the nature of the experiment. The cells suspended in 4ml of the same medium were transferred to tubes containing about $2\mu\text{mol}$ of yeast [^{32}P]phosphatidylcholine previously dispersed in 1ml of the same medium by vortex mixing for 30s with a glass bead. The suspension was gassed with O_2 -free CO_2 , sealed with a stopper and frozen in acetone/solid CO_2 . Incubation was carried out in water baths at various temperatures or in the freezing compartments of refrigerators or deep-freezers which had been stabilized for at least 24h before use. In certain experiments a steel Forbes bar drilled with holes (Johnson & Bangham, 1969) was used to obtain a temperature gradient. One end of the bar was maintained in a refrigerated ethylene glycol bath at -20°C , the other end in a water bath at 20°C .

Assay of phospholipase activity

Preparation of yeast [^{32}P]phosphatidylcholine and the determination of the water-soluble ^{32}P released from it on incubation, and the techniques for examination of the reaction products for glyceryl-phosphorylcholine and lysophosphatidylcholine have been described by Hazlewood & Dawson (1975).

Differential scanning calorimetry

Samples were sealed in aluminium micro-pans and the thermal-transition temperatures measured in a Perkin-Elmer differential scanning calorimeter

(model 1B) by using both the cooling and heating modes. Where appropriate, phosphatidylcholine was hydrated by mixing with an equal weight of water or incubation medium with the aid of a vortex mixer and allowing equilibrium to occur overnight. There is evidence that a higher proportion of water does not decrease the thermal-transition temperature further (Chapman, 1973).

Results

In early experiments it was observed that when yeast [32 P]phosphatidylcholine was mixed with a suspension of cells of the *Butyrivibrio* species in a buffer containing appropriate cofactors, substantial deacylation with the release of water-soluble phosphorus occurred at -16°C with the mixture frozen solid. No hydrolysis occurred at this temperature if the cell suspension was previously heated at 100°C for 15 min, suggesting that the reaction was enzymic. In comparison, less hydrolysis occurred at 39°C and the rate of this was not increased if the cells were kept frozen for 72 h at -16°C in the absence of substrate before assay at 39°C . This eliminated the possibility that a period of freezing was necessary to liberate the enzyme which then rapidly hydrolysed the substrate during the brief period of thawing before the extraction of the lipids with organic solvents. The degree of hydrolysis was also independent of the method used for terminating the reaction, e.g. chloroform/methanol (2:1, v/v); chloroform/methanol/6M-HCl (5:6:1, by vol.); precipitation with ice-cold 10% (w/v) trichloroacetic acid; or heating at 100°C for 2 min before chloroform/methanol treatment. This indicated that the solvent treatment used at the end of the reaction was not activating an enzyme liberated by the freezing. These results, together with the evidence that the reaction was time-dependent (see below), indicated that the hydrolysis was occurring while the cells and substrate were frozen solid in the incubation medium.

Effect of temperature on deacylase activity

Incubation of phosphatidylcholine with cells of the *Butyrivibrio* sp. at pH 6.8 and in the presence of 10 mM of both CaCl_2 and cysteine at a series of temperatures ranging from -16°C to 50°C revealed that maximum release of water-soluble P occurred around -10°C (Fig. 1). Virtually no hydrolysis could be demonstrated either at 4°C or -70°C , and appreciably less hydrolysis was demonstrated at the normal environmental temperature of the bacterium (39°C) when the incubation was carried out under the same conditions. However, considerably higher activity was observed at 39°C if potassium oleate or sodium dodecyl sulphate equimolar with the substrate was added to the medium to stimulate the activity

(Hazlewood & Dawson, 1975), and this enhanced breakdown occurred at a rate severalfold higher than that at -10°C . Addition of oleate caused an inhibition of the low-temperature deacylation, possibly by removing bivalent metal ions which seem to be stimulatory for the reaction. Since the commanding interest of the present observations was of a phospholipid-degrading enzyme system working in a solid medium, most of the subsequently described observations on the enzymes were carried

Fig. 1. Deacylation of phosphatidylcholine at various temperatures compared with thermal transitions of the hydrated substrate in incubation medium

For each temperature yeast [32 P]phosphatidylcholine ($47\text{ }\mu\text{g}$ of P) was incubated with bacterial cells (4.7 mg dry weight) in 5 ml of medium containing 0.1 M -dimethylglutaric acid/ NaOH buffer, 0.01 M -cysteine and 0.01 M - CaCl_2 . Tubes for incubation below 0°C were frozen in acetone/solid CO_2 and were then brought up to the required temperature for incubation for 23 h. The temperatures were obtained either by refrigerators and water baths (■) or by using a Forbes bar (●). Water-soluble ^{32}P released was assayed as stated in the methods. A further experiment carried out over 10 h showed essentially similar results with a maximum activity at -9°C . Differential scanning calorimetry was carried out on non-radioactive yeast phosphatidylcholine (18.9 mg) equilibrated overnight with an equal weight of the same incubation medium., Heat absorbed during a heating cycle in arbitrary units.

out at -16°C to ensure that the incubation medium was frozen solid.

Rate of formation and nature of products

On incubation of [^{32}P]phosphatidylcholine with the *Butyrivibrio* sp. cells at -16°C , the sole water-soluble ^{32}P -labelled product produced co-chromatographed on paper with a glycerylphosphorylcholine standard [phenol saturated with water/acetic acid/ethanol (100:10:12, by vol.) R_F 0.93; ethanol/13M- NH_3 solution (7:4, v/v) R_F 0.54]. The main ^{32}P -labelled lipid revealed by t.l.c. was unchanged substrate although small amounts of lysophosphatidylcholine could be detected at all times (Fig. 2). The breakdown of substrate was initially linear with no lag phase, but eventually hydrolysis stopped with a substantial part (40%) of the substrate remaining intact. At all times the amount of [^{32}P]glycerylphosphorylcholine formed greatly exceeded the lyso[^{32}P]phosphatidylcholine.

Action of thiol reagents

It was consistently observed that in the absence of cysteine the deacylation of phosphatidylcholine at -16°C was negligible (Table 1). Maximum stimulation of the reaction occurred at a cysteine concentration of 10mM although some activation was observed at 0.1mM. Comparison of cysteine with other thiol reagents all added at a concentration of 10mM showed that mercaptoethanol was approximately of equal effectiveness in stimulating deacylase, dithiothreitol somewhat less effective, and GSH was much less stimulatory (Table 1).

In its absolute requirement for thiol reagents the low-temperature activity of the enzyme system conforms with its requirements for such reagents observed at higher more physiological temperatures (Hazlewood & Dawson, 1975).

Fig. 2. Distribution of radioactivity during the incubation of bacterial cells with [^{32}P]phosphatidylcholine at -16°C

For each time-point the freshly harvested and washed cells (4.0mg dry weight) were incubated in 5ml of 0.1M-dimethylglutaric acid/NaOH buffer, pH6.8, containing 0.01M-cysteine, 0.001M- CaCl_2 and yeast [^{32}P]phosphatidylcholine (60 μg of P). All tubes were briefly gassed with O_2 -free CO_2 , immersed in acetone/solid CO_2 for 1 min and incubated at -16°C . After a two-phase solvent separation of the products, they were further purified by t.l.c. or paper chromatography and the distribution of radioactivity was determined. ●, Phosphatidylcholine; ■, lysophosphatidylcholine; ▲, glycerylphosphorylcholine. Results are representative of at least three experiments.

Table 1. Activation of low-temperature phospholipid deacylation by thiol reagents

The incubations were carried out in 5ml of 0.1M-dimethylglutaric acid/NaOH buffer, pH6.8. The contents of each tube were initially frozen by immersion in acetone/solid CO_2 for 60s. The thiol reagents were added at 10mM.

Incubation conditions		Substrate hydrolysed (μg of P/mg dry wt. of bacterial cells)				
		None	Cysteine	Dithiothreitol	Glutathione	Mercaptoethanol
Expt. 1	4.4mg dry wt. of bacterial cells; [^{32}P]phosphatidylcholine (61 μg of P); 1mM- Ca^{2+} ; 66h at -16°C	0.03	10.3	6.5	3.0	9.5
Expt. 2	3.95mg dry wt. of bacterial cells; [^{32}P]phosphatidylcholine (61 μg of P); 0.3mM- Ca^{2+} ; 0.375mM- Mg^{2+} ; 156h at -16°C	0.08	8.3	5.9	—	8.7
Expt. 3	4.3mg dry wt. of bacterial cells; [^{32}P]phosphatidylcholine (53 μg of P); 10mM- Ca^{2+} ; 25.5h at -5.5°C	<0.01	5.4	4.1	1.7	4.2

Effect of bivalent metal ions

Addition of Ca^{2+} to the system above a concentration of 0.1 mM produced in three experiments a stimulation which rose to a maximum at 10 mM (75–85%). The further addition of Ca^{2+} (1 M) produced a complete inhibition of the activity. Mn^{2+} and Mg^{2+} showed similar effects, although the activating effect of the latter tended to be marginally less. The addition of EDTA (1 mM) decreased the hydrolysis observed in the absence of added Ca^{2+} even further, but did not eliminate it entirely. Activations by Ca^{2+} were observed both at $-9\frac{1}{2}^{\circ}\text{C}$ and -16°C . The stimulation by bivalent metal ions seen at low temperatures could not be adequately tested at higher temperatures because of their interaction with the substances used to elicit enzymic activity, e.g. oleic acid or sodium dodecyl sulphate.

Effect of the physical state of medium and substrate on activity

The sudden and marked enhancement of deacylation activity obtained as the temperature of the present system was decreased below 0°C suggested that this might correspond to a phase change either in the hydrated yeast phosphatidylcholine itself or in the incubation medium. Op Den Kamp *et al.* (1974) have reported that the phospholipase A_2 (EC 3.1.1.4) of pig pancreas attacks various saturated phosphatidylcholine liposomes only near the transition temperature of the phospholipids. To examine this possibility in the present system, differential scanning calorimetry was carried out on yeast phosphatidylcholine both in the non-hydrated form, and when hydrated with either water or the incubation medium, as well as on the incubation medium itself. The transition temperatures were measured during the heating cycle to avoid complications due to supercooling. The thermal-transition temperature at which non-hydrated yeast phosphatidylcholine changed from the crystalline to the liquid-crystalline phase occurred at -33°C . On hydration of the phosphatidylcholine with either water or incubation medium the transition temperature was decreased (LT, Fig. 1) in conformity with results obtained with a fully saturated phosphatidylcholine (Chapman, 1973). Although this phase transition of the phospholipid in no way coincided with the marked enhancement of deacylation activity observed, there was, in contrast, a good correlation of peak enzymic activity with the phase change involved in melting of the incubation medium itself (IT, Fig. 1).

Since the increase in activity with decreased temperature in the present system more or less coincided with the freezing of the incubation medium the effect of adding ethylene glycol was investigated. Addition of 10% by volume, caused no phase change

in the frozen medium at -16°C and there was no diminution in deacylation. However, the addition of 10% by volume to the system at -5°C produced a liquefaction of the medium and a substantial (81%) inhibition of the deacylation. This would again suggest that the freezing of the medium was of importance in producing the enhancement of activity observed.

In addition, a sample of yeast [^{32}P]phosphatidylcholine was hydrogenated by using H_2 and an Adams platinum catalyst (Davenport & Dawson, 1962). Although substantial decomposition of phosphatidylcholine occurred it was possible to purify a sample by preparative t.l.c. and this contained almost entirely saturated acids (palmitic acid, stearic acid). Although the deacylation of this substrate was much less than that observed with normal yeast phosphatidylcholine, maximum activity was again well below 0°C . It is to be expected that the thermal-transition temperature of such a saturated phosphatidylcholine would be well above 40°C (Chapman, 1973).

In further experiments we observed the incubation mixture containing substrate and enzyme under phase-contrast light microscopy both at room temperature and after freezing the mixture on a cold stage. The freezing consistently produced a well-defined aggregation of substrate myelin figures and the bacterial cells which persisted even when the incubation mixture was warmed to room temperature (Plate 1).

Discussion

There is a good deal of evidence that enzymic hydrolysis of molecules can still occur in samples stored frozen at low temperatures (Albaum *et al.* 1952; Dawson *et al.*, 1957; Alford & Pierce, 1961), and as a consequence, the storage of edible plant and animal tissues at low temperatures leads to serious deterioration of quality unless the intrinsic enzymes are destroyed by brief heat treatment (blanching). Bengtsson & Bosund (1966) showed that the phospholipids in deep-frozen peas stored for 50–200 days, without a preliminary heat treatment, were deacylated. Similarly the phosphatidylcholine and phosphatidylethanolamine of frozen cod muscle are hydrolyzed by endogenous tissue enzymes (Olley & Lovern, 1960; Bligh, 1961). It has been generally assumed that such breakdowns represent very much lower enzymic activity compared with that observed at higher temperatures, with the temperature coefficient of the enzymes remaining positive until the lowest temperature that supports detectable activity is reached.

The existence of psychrophilic micro-organisms which can grow well at 0°C and proliferate at temperatures as low as -10°C is unequivocal proof

EXPLANATION OF PLATE I

Phase-contrast microscopy of substrate-bacterial mixtures

Stationary-phase washed bacterial cells (5.4mg dry wt.) and non-radioactive yeast phosphatidylcholine (66 μ g of P) were mixed together by vortex mixing for 30s with a glass bead, in 5ml of 0.1 M-dimethylglutaric acid/NaOH buffer, pH 6.8, containing 0.01 M-cysteine and 0.01 M- CaCl_2 . Portions were removed for microscopy on a Leitz ortholux photomicroscope fitted with an X97 oil immersion objective. The first portion was examined at room temperature. The second portion was left at -70°C for 15 min, and then allowed to warm to room temperature before being examined. (a) Before freezing. Myelin figures of substrate and dispersed bacterial cells are both visible. (b) After freezing. The bacterial cells completely surround the substrate particles. Magnification $\times 380$.

of the existence of enzymes capable of functioning efficiently at low temperatures (Stokes, 1968). However, the growth rate of such organisms decreases continuously with decreasing temperature right down to the minimum indicating that the temperature coefficients of the enzymes involved in the growth of the organisms are positive. The apparent 'negative temperature coefficient' of the present enzyme system as the temperature is decreased from 0° to 10°C is likely to be an artifact since it is associated with a phase change in the medium. This has the effect of bringing together the insoluble lipid substrate and membrane-bound enzymes so that the resulting initiation of attack surpasses any decreased efficiency of the enzymes due to the positive temperature coefficient. From the results obtained by differential scanning calorimetry, the addition of antifreeze and the hydrogenation of the substrate, it is clear that the low-temperature activity can be equated with the phase change in the incubation medium rather than that of the phospholipid substrate itself. It thus clearly differs from the activity of pancreatic phospholipase A₂ where at least for saturated lipids the breakdown is maximal near the transition temperature of the phospholipid substrate (Op Den Kamp *et al.*, 1974).

Sizer & Josephson (1942) and Sizer (1943) studied the activities of lipase, trypsin and invertase on decreasing the temperature and reported that there was a large change in the activation energy at about 0°C with a decrease in the hydrolysis rate. For lipase the activation energies were 154.8 kJ (37000 cal) below 0°C and 31.8 kJ (7600 cal) above this temperature. However, unlike the present system, the effect could not be correlated with a phase change in the incubation medium since similar results were obtained when freezing was prevented by the inclusion of high concentrations of glycerol.

The present results have implications for those concerned with the low-temperature storage of foods and other biological materials whether or not these may be contaminated with bacterial cells. Thus it is quite possible that freezing could cause a closer

aggregation or contact between a membrane-bound enzyme and its substrate in the cell leading to an enhanced decomposition unless the temperature was decreased very substantially below the freezing point of the tissue. Indeed some of the results obtained by Olley & Lovern (1960) strongly suggest that the low-temperature hydrolysis of phospholipids in cod muscle is enhanced by the freezing of the tissue. This is quite the reverse that might be predicted from the observed increase in activation energy reported for certain soluble enzymes at temperatures just below 0°C (Sizer & Josephson, 1942) and the usual activity/temperature kinetics.

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