THE INHIBITION OF *DICTYOSTELIUM DISCOIDEUM* ALKALINE PHOSPHATASE BY A LOW MOLECULAR WEIGHT FACTOR AND ITS IMPLICATION FOR THE DEVELOPMENTAL REGULATION OF THE ENZYME

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1. Introduction

During the terminal stages of the development of the cellular slime mold *Dictyostelium discoideum* there is a marked increase in the activity of the membrane-bound enzyme alkaline phosphatase [1,2], and evidence has been presented which suggests that this increase is due to de novo enzyme synthesis [1]. However, in a recent report from this laboratory, it was shown that the alkaline phosphatase activity of vegetative cells is markedly activated by incubating membranes at 50°C prior to assay, and inactivated by incubating membranes at 0°C, both phenomena being totally reversible [3]. In contrast, the activity of culminating cells is neither activated at 50°C, nor inactivated at 0°C. These results suggest that the increase in activity during development may be due to the unmasking of existing enzyme, a phenomenon that is simulated by the 50°C treatment. Alkaline phosphatase solubilized from the membrane by butanol treatment is neither activated at 50°C nor inactivated at 0°C [3] suggesting that membrane integrity is in some way involved in the suppression of alkaline phosphatase activity in vegetative cells.

The experiments described in this report demonstrate that the alkaline phosphatase activity of vegetative cells is suppressed by a membrane bound inhibitor, which is removed from the membrane as development progresses.

2. Materials and methods

Dictyostelium discoideum Ax-2, an axenic strain was grown in HL-5 medium as previously described [4]. Cells were harvested at a cell density of 5×10^6 cells ml^{-1} by centrifugation at $700 \times g$ for 2 min. For differentiation experiments the pellets were washed once with cold deionised water and resuspended in the same at a cell density of 1.5×10^8 cells ml^{-1}. Aliquots (0.3 ml) were spread onto Whatman No. 50 filter papers (4.25 cm) soaked in lower pad solution [5]. The filters were incubated at 22°C and observed periodically. The time course of differentiation under these conditions was the same as described previously [6]. Cells from the pseudoplasmodial and early and late culminating fruiting body stages of development were washed from the filter with 5 mM Tris–HCl buffer (pH 7.4) containing 8.6% sucrose.

Crude membranes were prepared essentially as described previously [7]. Harvested cells were washed again in 5 mM Tris–HCl (pH 7.4) containing 8.6% sucrose and resuspended in the same buffer saturated with PMSF at a cell density of 10^8 cells ml. The cells were disrupted by mechanical grinding with glass beads (more than 90% disruption) and separated from unbroken cells and glass beads by centrifugation at $700 \times g$ for 5 min. The supernatant was centrifuged at $105\,000 \times g$ to pellet the crude membrane fraction which was resuspended with 5 mM Tris–HCl buffer (pH 7.4) containing 8.6% sucrose and recentrifuged. The final pellet was resuspended in a small volume of the same buffer to give a final protein concentration of about 25 mg ml^{-1}. In some experiments samples were dialyzed against 20 times the volume of 5 mM Tris–HCl (pH 7.5). For extended dialysis experiments, the buffer was replaced every 24 h.

Alkaline phosphatase was assayed as described previously [2], with the addition of 30 mM sodium fluoride to inhibit acid phosphatase. Protein was determined by the Folin procedure [8].

3. Results and discussion

In order to more fully understand the previously reported activation of alkaline phosphatase at 50°C and inactivation at 0°C, purification of the enzyme was initiated. Membranes were subjected to a variety of treatments to determine the best procedure for extracting alkaline phosphatase activity. Butanol and Triton X-100 solubilized a large proportion of the alkaline phosphatase activity and both treatments increased the total enzyme activity (table 1). The butanol extract was also considerably enriched in terms of specific activity (table 1). Sodium deoxycholate and sodium dodecyl sulphate were less efficient in releasing alkaline phosphatase from the membrane and both detergent treatments decreased the total enzyme activity. Treatment of the membrane with 3 M KCl or 20 mM EDTA failed to release any activity (data not shown), suggesting that alkaline phosphatase is firmly bound to the membrane by hydrophobic interactions.

The alkaline phosphatase activity of the Triton X-100 extracted material was stimulated by incubation at 50°C and inhibited by subsequent incubation at 0°C (fig.1). A second incubation at 50°C reversed

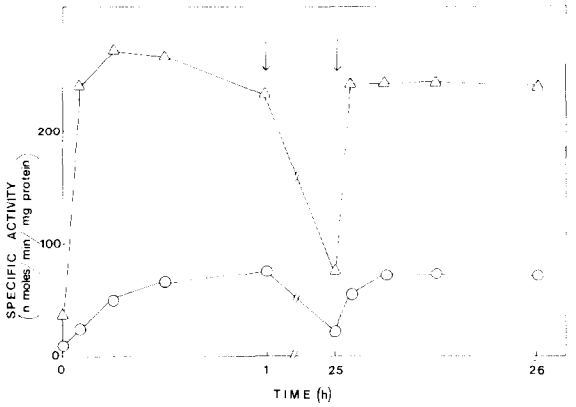

Fig.1. Effect of 50°C and 0°C incubation on the alkaline phosphatase activity of intact membranes and Triton X-100 extracted membranes. Intact membranes at 4.0 mg protein ml^{-1} (○) and Triton X-100 extracts of membranes at 1.5 mg protein ml^{-1} (△) in 5 mM Tris–HCl pH 7.4 were incubated at 50°C and, at the indicated time points, 0.1-ml aliquots were assayed for alkaline phosphatase activity. At the first arrow, the samples were transferred to 0°C and incubated for 24 h. At the second arrow the samples were transferred back to 50°C and 0.1 ml aliquots assayed at the indicated points for alkaline phosphatase activity

Table 1
Comparison of various procedures for the extraction of alkaline phosphatase from vegetative cell membranes of *D. discoideum*

Treatment	Total activity (nmol min^{-1})		Specific activity (nmol min^{-1} mg^{-1})	
	membrane	supernatant	membrane	supernatant
None	432	0	8.8	0
Butanol[a]	494	1296	16.6	74.0
Triton X-100 (1.0%)[b]	167	833	4.0	17.5
Sodium deoxycholate (1.0%)[b]	136	123	7.2	2.6
Sodium dodecyl sulphate (0.1%)[b]	299	0	6.5	0

[a] 0.4 ml butanol was added to 1.0 ml of crude membrane suspension in 5 mM Tris–HCl, pH 7.4 (10 mg protein ml^{-1}) and vortexed intermittently for 1 h. The aqueous phase was separated and centrifuged at 35 000 × *g*, for 30 min. Both pellet (membrane) and supernatant were dialyzed to remove butanol and then assayed for alkaline phosphatase activity

[b] Crude membranes resuspended at 20 mg protein ml^{-1} in 0.01 M Tris–HCl, pH 7.4 were incubated with the detergents for 2 h at 4°C with occasional stirring. This was centrifuged at 105 000 × *g* at 4°C for 75 min. The pellet (membrane) was resuspended in 0.01 M Tris–HCl buffer (pH 7.4), and both the pellet and supernatant were assayed for alkaline phosphatase activity

the inhibition (fig.1). This result is comparable to that reported previously [3] for the alkaline phosphatase activity of the intact membrane (see also fig.1). In contrast, butanol-extracted alkaline phosphatase activity was inhibited by incubation at 50°C [3]. Thus, it is apparent that membrane integrity is not essential for the 50°C activation and 0°C inactivation of the alkaline phosphatase, as previously suggested [3].

Preliminary studies on the purification of alkaline phosphatase revealed that the butanol solubilized material was totally excluded from a Sephacryl S-300 column (data not shown), suggesting that the solubilized protein was in the form of large aggregates. In contrast, there was a partial separation of the Triton X-100 solubilized material on Sephacryl S-300, and the total activity recovered from the column was markedly enhanced (data not shown), suggesting that an inhibitory molecule was removed from the enzyme by the gel filtration procedure.

Overnight dialysis of the Triton-solubilized enzyme yielded a 10-fold increase in enzyme activity (table 2). This activation was comparable to that observed during gel filtration suggesting that the inhibitory molecule removed on the column was of low molecular weight. The alkaline phosphatase activity of intact membranes was also activated almost 10-fold by dialysis (table 2), but this level of activation was only attained after three days of continuous dialysis, suggesting that the low molecular weight inhibitor was tightly bound to the membrane. The specific activity of the dialyzed intact membrane was comparable to that of the butanol extracted activity (table 1), which was exhaustively dialyzed to remove the inhibitory effects of butanol, prior to assay. These activities are also comparable to the maximum activity obtained as a result of 50°C incubation of intact membranes

Table 2
Effect of dialysis and subsequent 50°C treatment on alkaline phosphatase of membranes and Triton X-100 extracts

	Specific activity of alkaline phosphatase (nmol min^{-1} mg^{-1})		
	Before dialysis	After dialysis	Incubation at 50°C for 1 h after dialysis
Crude membrane	9.5	100	123.4
Triton extract	37.0	370	323.3

(fig.1 and [3]) suggesting that 50°C treatment may activate the enzyme by releasing the inhibitor. This suggestion was corroborated by the finding that there was essentially no change in the activity of the dialyzed intact membrane or the dialyzed Triton X-100 extracted enzyme upon incubation at 50°C (table 2). Thus it is apparent that incubation at 50°C, dialysis and column chromatography, all enhance the activity of alkaline phosphatase by removing a low molecular weight inhibitor from the enzyme.

Intact crude membranes from cells of the vegetative, pseudoplasmodial and early and late culmination phases of development were dialyzed and assayed for alkaline phosphatase activity. As shown in table 3, dialysis results in only a slight stimulation of the activities of the enzymes from the terminal stages of differentiation. The pseudoplasmodial enzyme was activated two-fold (table 3). These results are in marked contrast to the 12-fold increase in the activity in the dialyzed crude membrane of vegetative cells (table 3). The data presented in table 3 also show that the alkaline phosphatase activities of the dialyzed membranes from four developmental stages are similar, indicating

Table 3
Effect of dialysis on the specific activity of alkaline phosphatase of membranes prepared from Ax-2 cells at various stages of development

Developmental stage	Developmental time (h)	Specific activity of alkaline phosphatase (nmol min^{-1} mg^{-1})	
		non-dialyzed[a]	dialyzed[b]
Vegetative	0	9.2	108.0
Pseudoplasmodium	22	43.0	86.0
Early culmination	30	108.0	129.0
Late culmination	34	105.0	126.0

[a] Freshly prepared membranes
[b] Membrane preparations dialyzed for three days at 4°C

that the apparent developmentally regulated increase in the activity of alkaline phosphatase in undialyzed preparations (table 3) is due to the removal of the low molecular weight inhibitor.

Several years ago, Gezelius and Wright reported differential instability of alkaline phosphatase extracted from vegetative amoebae, preculmination cells and sorocarps [9]. Enzyme from vegetative cells retained only ten percent of its activity when cell-free extracts were stored for 8 days at 4°C, while from preculmination cells and sorocarp cells retained 29% and 50% of the initial activity respectively under identical conditions. Despite the differences in experimental procedure, these results are similar to our recent findings on the temperature dependent activation and inactivation of this enzyme [3]. The stability of the alkaline phosphatase from cells of late developmental stages in the earlier study [9] might be explained by the presence of only low levels of inhibitor in these membranes. Gezelius and Wright argued that inhibition of the enzyme by orthophosphate might play an important regulatory role during development. The available evidence indicates that the low molecular weight inhibitory molecule described in the present report is not phosphate. Phosphate inhibition of alkaline phosphatase can not be reversed either by dilution, dialysis or incubation of the membrane preparation at 50°C (data not shown). The molecular nature of the inhibitory molecule remains to be determined.

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