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Release of Enzymes by Normal and Wall-Free Cells of *Chlamydomonas*

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The phosphatases produced by the wild-type strain of *Chlamydomonas reinhardi* in media deprived of inorganic phosphate are found partly inside and partly outside the cells. The same enzymes are almost completely released by a mutant strain defective in cell wall formation. It is proposed that the failure of cell wall mutants to survive in certain conditions is related to their inability to retain certain essential compounds that are normally associated to the cell wall.

Numerous studies of enzyme localization in *Escherichia coli* and other gram-negative bacteria have accumulated evidence for the presence of certain enzymes in a region between the cytoplasmic membrane and the cell wall (3). These enzymes (namely alkaline phosphatase, 5'-nucleotidase, and ribonuclease) are normally associated with the cells but can be selectively released by an osmotic shock or by conversion of the cells to spheroplasts. In yeast, large amounts of invertase (12), acid phosphatase, and β-fructofuranosidase (1), predominantly attached to the cell wall in normal cells, are released into the medium during protoplast formation. In *Chlamydomonas reinhardi*, stable mutants deprived of cell wall have been isolated (2); they can be considered as permanent protoplasts and aid the investigation of the physiological role of the cell wall.

The aim of the present study was to compare the release of certain proteins from *C. reinhardi* cells having a normal cell wall (wild type, WT) and cells deprived of cell wall (2). The phosphatases were particularly suitable for this work: considerable phosphatase activities were previously detected in the growth media of wild type and certain specific mutants (4, 6). The different *Chlamydomonas* phosphatases have been well characterized (11): two constitutive acid phosphatases (constitutive soluble [CS] and constitutive insoluble [CI]) are produced in cells grown on media containing high concentrations of inorganic phosphate, and three additional derepressible phosphatases (one neutral, DN; two alkaline, DA_1 and DA_2) are synthesized on media deprived of inorganic phosphate. Mutants defective in CI (7), CS (7), and DN (10) are available.

It was possible to investigate separately the fate of the different phosphatases by utilizing various mutants and taking into account the properties of each enzyme (derepressibility, pH range for activity, and thermosensitivity).

MATERIALS AND METHODS

Strains. The different strains used in this study are described in Table 1.

Media. The cells were grown at 25°C in continuous light (5,000 lx) on solid media, which support much better than liquid media the growth of cell wall mutants. The culture media were modifications of the tris(hydroxymethyl)aminomethane (Tris)-minimal phosphate medium given by Surzycki (13). (+P) medium contained 5 mM P_i; the (−P) medium was Tris-minimal phosphate medium deprived of inorganic phosphate.

Preparation of extracts and secretions. It was found that cellophane membranes interposed between the agar and the cells were permeable to cytochrome *c* (molecular weight, 12,500) and chymotrypsinogen A (molecular weight, 25,000) but not to egg white albumin (molecular weight, 45,000) and other proteins of higher molecular weight. This observation suggested a simple but efficient method for collecting from small cultures relatively concentrated solutions of proteins secreted by the cells. All *Chlamydomonas* phosphatases were retained on the membranes.

Two days after pouring the medium, sterile cellophane membrane squares (6.5 by 6.5 cm) were laid down on the plates. About 10^5 cells were immediately spread over the surface of each membrane. After 4 to 5 days of incubation, a uniform green carpet of cells developed. The membranes were picked up, and the cells were resuspended in a small volume of 0.02 M Tris-hydrochloride buffer (pH 7.0, about 1.5 ml/membrane) and centrifuged at 27,000 × *g* for 15 min. The supernatant contained the compounds secreted by the cells. The cell pellet was washed twice, resuspended in water, and disintegrated with ultrasound (MSE ultrasonic disintegrator). Cellular debris was eliminated by centrifugation (27,000 × *g*, 15 min), and the supernatant was used as a crude extract.

Assays. The phosphatases were assayed as described previously (7) using naphthyl phosphate as a substrate and tetrazotized-o-dianisidine as a diazonium agent. Protein was measured according to the method of Lowry et al. (8) using egg white lysozyme as a standard.

RESULTS AND DISCUSSION

To verify whether constitutive acid phosphatases (CS and CI) were released by WT or by CW_{15}, the two strains were grown on (+P) medium, after which the phosphatase activities were measured in cell extracts and secretions (Table 2).

In all experiments performed on (+P) or (−P) medium, the secretions of CW_{15} contained two to five times more protein than those of WT.

In CW_{15} about 20% and in WT about 2% of the enzyme units were found in the secretions. The specific activities in both strains were lower in secretions than in cell extracts. On the basis of CI resistance to heat (10), it was possible to decide which of the two enzymes (CI or CS) was lost by CW_{15}. It was found that the CW_{15} secretions retained 86% of their acid phosphatase initial activity after a 10-min treatment at 60°C, which strongly suggests that the constitutive phosphatase released by CW_{15} corresponds to CI and that this enzyme could be located at least partially near the cell wall. Our attempts to localize CI cytochemically have not been successful. The CS enzyme that is undoubtedly localized in vacuoles (11) was probably fully retained by WT and CW_{15} cells.

The derepressible phosphatases were studied after growing various mutant strains on (−P) medium. The DN phosphatase was assayed in P_2P_a and $P_2P_aCW_{15}$ cells at pH 4.8 (under these conditions, the DA phosphatases are almost inactive). The two alkaline phosphatases, which are virtually indistinguishable, were assayed together at their pH optimum (pH 9.5 glycine-sodium hydroxide buffer) from P_2P_aPD mutants lacking all other phosphatases (Table 3). (i) Cells having a normal cell wall retained a significant part of the total phosphatase (65% of DN, 23% of DA). (ii) In mutants deprived of cell wall, only traces of these enzymes were found associated to the cells, most phosphatases being released outside the cells. (iii) The total phosphatase activity in $P_2P_aPD_4CW_{15}$ was very low (a value close to what was found in $P_2P_aPD_4$ was expected), indicating that the absence of cell wall could interfere with the synthesis or lead to increased breakdown of the DA phosphatases.

The DN phosphatase has been cytochemically localized at the level of the cell wall (11), and it is then understandable why this enzyme escapes from the CW_{15} cells. DA_1 and DA_2 were not cytochemically visualized (11), but the present data suggest that they are also associated to the cell wall. The release of derepressible phosphatases is selective. That is, the specific activities were always higher in secretions than in cell extracts whether the cell wall was present or not. The almost complete leakage of dere-

TABLE 1. *Description of strains*

Designation	Characteristics	Origin
WT (137 C)	WT strain	R. P. Levine (Harvard)
CW_{15}	Mutant deprived of cell wall	Davies and Plaskitt (2)
P_2P_a	Mutant impaired in the activity of both constitutive phosphatases	Loppes and Matagne (7)
$P_2P_aPD_4$	Mutant impaired in the activity of both constitutive phosphatases and of the neutral derepressible phosphatase	Matagne and Loppes (10)
$P_2P_aCW_{15}$	P_2P_a lacking the cell wall	Product of cross $CW_{15} \times P_2P_a$
$P_2P_aPD_4CW_{15}$	$P_2P_aPD_4$ lacking the cell wall	Product of cross $CW_{15} \times P_2P_aPD_4$

TABLE 2. *Phosphatase distribution after growth on phosphate-containing medium*[a]

Strain	Enzymes assayed	Protein/plate		Phosphatase/plate (μmol of naphthol/h, 37°C)		Sp act of phosphatases (μmol of naphthol/mg of protein per h, 37°C)	
		Inside	Outside	Inside	Outside	Inside	Outside
WT	CS + CI	2.33	0.15	1.11	0.02	0.48	0.12
CW_{15}	CS + CI	2.16	0.57	1.00	0.20	0.47	0.36

[a] Phosphatase units (micromoles of naphthol per hour at 37°C) per plate, protein per plate, and phosphatase specific activities (micromoles of naphthol per milligram of protein per hour at 37°C) in cell extracts (inside) and secretions (outside) from WT and CW_{15} cells grown on (+P) medium for 5 days. All assays were carried out at pH 4.8 (acetate buffer).

TABLE 3. *Phosphatase distribution after growth on phosphate-deficient medium*[a]

Strain	Cell wall	pH of assay	Enzyme(s) assayed	Protein/plate		Phosphatase units/plate (μmol of naphthol/h, 37°C)		Sp act of phosphatases (μmol of naphthol/mg of protein per h, 37°C)	
				Inside	Outside	Inside	Outside	Inside	Outside
P_2P_a	+	4.8	DN	1.10	0.26	18.98	10.37	17.42	37.50
$P_2P_aCW_{15}$	−	4.8	DN	1.85	0.48	1.21	37.25	0.66	77.30
$P_2P_aPD_4$	+	9.5	$DA_1 + DA_2$	2.50	0.24	2.69	8.94	1.09	33.30
$P_2P_aPD_4CW_{15}$	−	9.5	$DA_1 + DA_2$	2.16	0.62	0.004	2.22	0.002	3.62

[a] Phosphatase units (micromoles of naphthol per hour at 37°C) per plate, protein per plate, and phosphatase specific activities (micromoles of naphthol per milligram of protein per hour at 37°C) in extracts (inside) and secretions (outside) of WT and CW_{15} cells grown on (−P) medium for 5 days.

pressible phosphatases in CW_{15} was accompanied by the release of other proteins, one of which has been identified as α-naphthylesterase (R. Loppes, results not shown).

The leakage of phosphatases and other enzymes by mutants having cell wall defects was reported in bacteria (5, 9). Certain of these mutants were lethal, except when exposed to high osmotic pressures. Many cell wall mutants of *Chlamydomonas* fail to survive in liquid media and on solid media when plated at low cell concentration (2, 6). Their survival is not favored by high concentrations of various sugars, which indicates that they are not osmotic remedial (2).

As shown in this report, certain proteins are almost completely lost by the CW_{15} mutant. The loss of phosphatases should not be dramatic for the cell, at least in media containing enough inorganic phosphate, but it might be that other compounds associated to the cell wall are essential to growth.

We observed that the plating efficiency could be greatly enhanced when CW_{15} was plated together with a high number of auxotrophic cells. In a typical experiment, few CW_{15} cells were plated on minimal medium either alone or mixed with about 10^7 *arg-1* cells. Two colonies per plate were obtained when CW_{15} was plated alone, whereas 240 colonies developed on the plates carrying the carpet of dying *arg-1* cells. The survival of CW_{15} could be due to the fact that it receives from *arg-1* cells certain compounds of physiological importance that have been lost by CW_{15} during the preparation of the plating suspension. These compounds could be specific proteins or nucleic acid-related substances (the ultraviolet absorption spectra of WT and CW_{15} secretions displayed a maximum at 269 nm and a minimum at 243 nm, which is far from being characteristic of pure protein solutions) indispensable to the development of the alga.

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