Identification and Purification of a Derepressible Alkaline Phosphatase from *Anacystis nidulans* R2

**ABSTRACT**

We have examined the increase in alkaline phosphatase activity in the cyanobacterium *Anacystis nidulans* R2 upon phosphate deprivation. Much of the activity is released into the medium when *A. nidulans* is osmotically shocked, indicating that the enzyme is located either in the periplasmic space or is loosely bound to the cell wall. The polypeptide associated with phosphatase activity has been identified as a single species of M, 160,000. Several lines of evidence demonstrate that this polypeptide is responsible for alkaline phosphatase activity: (a) It is absent when cells are grown in the presence of phosphate and specifically accumulates during phosphate deprivation. (b) It is the major periplasmic polypeptide extracted by osmotic shock. (c) It represents over 90% of the protein in a fraction of periplasmic polypeptides enriched for phosphatase activity. (d) Antibodies raised against the purified species of M, 160,000 inhibit phosphatase activity by approximately 70%.

Phosphate, an essential nutrient for all organisms, is present in nucleic acids, phospholipids, and a number of biochemically important solutes such as ATP. It is required by cells in large amounts, frequently limits the growth of both terrestrial and aquatic organisms, and is actively transported into most organisms as the phosphate anion. During periods of phosphate limitation many microorganisms employ emergency systems which aid in its acquisition. *Anacystis nidulans* (Synechococcus 6301), a unicellular cyanobacterium, exhibits marked physiological and biochemical changes when deprived of phosphate. Following deprivation, growth stops and the cells become bleached; the light harvesting phycobilisome complex is degraded, Chl levels decline, and cyanophycin granules accumulate (21). In addition, both acid soluble and insoluble phosphorus pools begin to decline and phosphate uptake is significantly enhanced (8). One of the most well documented changes that occurs in this microorganism during phosphate deprivation is the appearance of an alkaline phosphatase (12).

The production of alkaline phosphatase in response to phosphate limitation has been extensively examined in *Escherichia coli* (23, 26) and several different prokaryotic (11) and eukaryotic (28) organisms. The enzyme is a nonspecific phosphomonoesterase generally associated with zinc. In several organisms much of the alkaline phosphatase activity is localized to the periplasmic space. Mechanisms involved in regulated expression of alkaline phosphatase activity have been examined in *E. coli* (22, 24), *Bacillus licheniformis* (11), and the yeast *Saccharomyces cerevisiae* (28). In *E. coli* the alkaline phosphatase gene is part of the complex pho regulon which is controlled by the single repressor substance inorganic phosphate (22, 24). The genes of the pho regulon are scattered on the *E. coli* chromosome and the products of these genes are both structural elements required for elevated phosphate acquisition and regulatory elements involved in transcriptional control. While both genetic and biochemical approaches have increased our understanding of several events which accompany phosphate limitation and the functions of certain polypeptides encoded by member genes of the pho regulon, many aspects of the regulation are still unclear. In the eukaryotic organism *S. cerevisiae* (1, 28) the genes involved in acclimation to low phosphate conditions are also dispersed on the genome and encode numerous structural and regulatory elements. As in *E. coli*, elevated synthesis of alkaline phosphatase in this organism during phosphate deprivation has been used to analyze the molecular control of gene regulation during acclimation.

In this paper, we confirm the appearance of an alkaline phosphatase activity in the cyanobacterium *A. nidulans* during phosphate deprivation and demonstrate that much of the activity is localized to the periplasmic space. Furthermore, the polypeptide responsible for the phosphatase activity was purified from periplasmic proteins extracted from phosphate-starved cells. Antibodies prepared against the denatured monomer were used to confirm the identification of the enzyme.

**MATERIALS AND METHODS**

**Culture Conditions.** *Anacystis nidulans*, strain R2, was grown to mid-logarithmic phase (2–5 × 10⁶ cells/ml) at 30°C in BG-11 medium (20) bubbled with 3% CO₂ in air. Growth was in continuous light at an intensity of 100 μmol m⁻² s⁻¹. To achieve phosphate deprivation, cells were harvested by centrifugation at 3500g for 5 min, washed in BG-11 medium in which KH₂PO₄ was replaced by KCl (phosphate-free medium) and then resuspended in phosphate-free medium at a 3 to 4 times lower cell density.

**Measurement of Phosphatase Activity.** Either whole cell suspensions (unlysed) or preparations of periplasmic proteins (see below) were made to 0.2 mM Tris-HCl (pH 8.5), 2 mM MgCl₂, and 3.6 mM p-nitrophenyl- P (Sigma) (final volume 1.5 ml), unless otherwise specified, and incubated at 37°C for 5 to 15 min. The reaction was stopped with 150 μl of 4 N NaOH, the mixture centrifuged 4 min in an Eppendorf microfuge (7000g), and the absorbance of the supernatant measured at 400 nm and compared to a standard absorbance curve for p-nitrophenol (Sigma). Under these conditions the reaction was linear with respect to both time and enzyme concentration.

**Extraction of Total Soluble Proteins.** Cells from approximately 50 ml of culture were harvested by centrifugation at 3500g for 5 min and resuspended in 6 ml of ice-cold 5 mM Tris-HCl (pH 7.5), 1 mM benzamidin-HCl, 1 mM phenylmethylsulfonylfluor-
ide, 1 mM \( \varepsilon \)-aminocaproic acid. Cells were lysed in a chilled French pressure cell at 100 MPa and unbroken cells removed by centrifugation for 10 min at 3000g. The supernatant was made 100 mM NaCl and centrifuged 30 min at 27,000g to eliminate most of the thylakoid membranes.

**Extraction of Periplasmic Fraction.** The periplasmic fraction was prepared from 300 ml of cells at 3 to 6 \( \times 10^9 \) cells/ml. Cells were harvested by centrifugation at 3500g for 5 min and resuspended in 10 ml of 40% sucrose with 0.2 mM MgCl\(_2\), 2 mM EDTA. After 30 min of gentle agitation at room temperature, the cells were pelleted by centrifugation at 8000g for 10 min, and resuspended in 5 ml of ice-cold 5 mM Tris-HCl (pH 7.5). After a 10 min incubation on ice, the suspension was centrifuged at 6000g for 5 min and the supernatant collected and made 3 mM MgCl\(_2\), 1 mM benzamidine-HCl, 1 mM phenylmethylsulfonylfluoride, and 1 mM \( \varepsilon \)-aminocaproic acid. Extraction of periplasmic proteins was sometimes repeated on the same cell suspension.

**Partial Purification of Alkaline Phosphatase.** The periplasmic extract (2–3 mg protein) was layered onto a 10 to 60% sucrose gradient (36 ml/tube) in 20 mM Tris-HCl (pH 7.5), 2 mM MgCl\(_2\), and centrifuged 19 h at 76,000g at 4°C. Following this centrifugation the gradient was fractionated into 2.4 ml aliquots which were assayed for phosphatase activity. Approximately 75% of the proteins remained at the top of the gradient while a single peak of phosphatase activity was located near the middle of the gradient. The four fractions containing the highest activity were pooled and dialyzed 12 h in 5 mM Tris-HCl (pH 7.5), 1 mM MgCl\(_2\). After the addition of Triton X-100 (to 0.05%) to the dialyzed material, it was layered on a DEAE-cellulose (Sigma) column (0.8 ml of gel) equilibrated with buffer A (5 mM Tris-HCl [pH 7.5], 1 mM MgCl\(_2\), 0.05% Triton X-100) and allowed to flow through the column at 12 ml/h. The column was washed with 8 ml of buffer A prior to elution of the phosphatase activity using a 20 ml linear gradient of 0 to 0.4 M NaCl in buffer A. Fractions of 1.5 ml were collected and assayed for phosphatase activity. All purification steps were performed at 4°C.

**Electrophoresis of Polypeptides and Determination of Protein Concentration.** Proteins were precipitated on ice in 10% trichloroacetic acid for 2 h, pelleted by centrifugation at 12,000g for 30 min at 4°C, washed once with ice-cold 90% acetone, and resuspended by sonication in 0.1 M Na\(_2\)CO\(_3\), 0.1 M dithiothreitol to a concentration of between 1 and 5 mg protein/ml. The suspensions were made 1.67% SDS by adding 0.5 volume of 5 M guanidine hydrochloride. Proteins were precipitated on ice in 6 M guanidine hydrochloride and resuspended in 8 ml of buffer A prior to elution of the phosphatase activity. All steps were performed at 4°C.

**Preparation of Antibodies.** Polypeptides present in the partially purified alkaline phosphatase fraction were separated by SDS-PAGE. The major polypeptide (M, 160,000) was excised from the gel, electroeluted, combined with Freund's adjuvant, and injected into a rabbit following an immunization schedule previously described (4). The IgG fraction was purified from the rabbit serum by chromatography over DEAE-affigel Blue (BioRad) as described by the manufacturer and concentrated by ammonium sulfate precipitation (25 g/ml).

**Western Blot Analyses.** Polypeptides separated on a polyacrylamide gel were transferred to nitrocellulose paper by electrophoresis (25). The nitrocellulose sheet was incubated 2 h at 20°C in TS (10 mM Tris-HCl pH 7.4/170 mM NaCl) with 30 g/l BSA and then overnight at 4°C with IgG (0.2 \( \mu \)g protein/ml) in TS-BSA. Nonspecific binding of the antibodies to the nitrocellulose was eliminated by several washings with TS. The nitrocellulose sheet was then incubated 1 h at 20°C in TS-BSA and 2 h at 20°C with protein A-peroxidase (Boehringer Mannheim, West Germany) 27 \( \times 10^{-3} \) units/ml in TS-BSA. Excess protein A-peroxidase was removed from the filters by several washings in TS and the antigen-antibody-protein A-peroxidase complex was stained according to Haid and Suisa (9) using 4-chloro-1-naphtol (Sigma) as substrate.

**RESULTS**

**Phosphatase Accumulation.** As shown in Figure 1, the transfer of *Anacystis nidulans* from medium containing 0.23 mM phosphate to phosphate-free medium results in an increase in alkaline phosphatase activity, as measured in suspensions of intact cells. The lag phase preceding the accumulation is less than 1 h. After 12 h of phosphate deprivation, the total alkaline phosphatase activity of the cultures becomes elevated four- to fivefold. When the cells are deprived of sulfate instead of phosphate, no increase in phosphatase activity is detected even after 24 h of starvation (result not shown).

Since alkaline phosphatase activity is readily measured in cell suspension, it was likely that the enzyme was localized to the periplasmic space, as in *E. coli* (19). To confirm this we isolated...
periplasmic proteins using an osmotic shock procedure (see "Materials and Methods") commonly used for E. coli. It involves plasmolyzing bacterial cells in sucrose in the presence of EDTA to permeabilize the outer membrane, followed by a rapid dilution into cold low osmoticum buffer to expel periplasmic proteins (6). Almost no alkaline phosphatase activity is present in the periplasmic fraction from cells grown in the presence of phosphate while elevated levels are readily detected 4 h after the cells are transferred to phosphate-free medium and maximal activity (15–20-fold increase) is attained after 12 h. At 24 h, the activity decreases slightly, probably because the cells become fragile and may release proteolytic enzymes during the extraction of periplasmic proteins.

Analysis of Periplasmic Polypeptides. The analyses of periplasmic polypeptides by SDS-PAGE (Fig. 2) show the appearance of a major polypeptide with a M, of 160,000 which specifically accumulates during phosphate deprivation. Other minor species which migrate just below the M, 160,000 polypeptide, also appear. The M, 160,000 polypeptide, detected by 4 h after depriving the cells of phosphate (Fig. 2, lane 2), becomes a prominent polypeptide in the periplasmic fraction 6 to 8 h after removal of the nutrient from the medium (Fig. 2, lane 3). The pattern of polypeptides which migrate just below the M, 160,000 species is variable as are the levels of each. This variability appears to depend on the age of the culture used in the experiment. These polypeptides probably represent proteolytic breakdown products of the alkaline phosphatase (discussed below). Other polypeptides in the periplasmic fraction become less abundant after the transfer (e.g. M, 42,000 and 37,000 polypeptides).

Properties of the Phosphatase. We have investigated the kinetic properties of the alkaline phosphatase using the periplasmic fraction from cells deprived of phosphate for 24 h. The enzyme is inhibited by EDTA, but the inhibition can be partially reversed by the addition of Mg\textsuperscript{2+} (Fig. 3A). MgCl\textsubscript{2} itself stimulates the activity slightly. In contrast, ZnCl\textsubscript{2} inhibits the activity (not shown). The enzyme has a broad pH optimum over the range of pH 8.5 to 9.5 (Fig. 3B) in good agreement with what has already been reported (12). Figure 3C shows a double reciprocal plot from which a K\textsubscript{m} for p-nitrophenyl phosphate of 2.6 mM is derived.

![Fig. 2. SDS-polyacrylamide gel of polypeptides in the periplasmic fraction of A. nidulans after transfer to phosphate-free medium. Cells were grown in phosphate-free BG-11 medium for 0, 4, 8, 12 h (lanes 1, 2, 3, 4, respectively) prior to the extraction of periplasmic proteins.](image)

![Fig. 3. Enzymatic properties of the periplasmic alkaline phosphatase. A, Effect of MgCl\textsubscript{2}, in the presence (○) and absence (□) of 5 mM EDTA, on phosphatase activity. B, Phosphatase activity as a function of pH in 0.2 M Tris-glycine buffer. C, Determination of the K\textsubscript{m} for p-nitrophenyl phosphate in a double reciprocal plot. In each case the phosphatase activity was measured using the periplasmic fraction from cells deprived of phosphate for 24 h. 8 \mu g of protein were used for each measurement.](image)
This value is comparable with the value reported for _E. coli_ alkaline phosphatase (10).

**Phosphatase Purification.** To identify the polypeptide(s) responsible for phosphatase activity, we prepared periplasmic fractions from cells starved for phosphate for 24 h. While the specific activity of the phosphatase in this fraction is high, only 20 to 30% of the total cellular activity is recovered. Further purification of the phosphatase was achieved by fractionation on a sucrose gradient (Fig. 4). Phosphatase activity is recovered from the gradient at 35 to 40% sucrose after 19 h of centrifugation at 76,000g. The specific activity increased three- to fourfold and this fraction is enriched in the M, 160,000 polypeptide (Fig. 5, lane 2). Other high mol wt polypeptides are also enriched, while the small polypeptides (M, < 33,000) are completely eliminated. After dialysis, the fractions containing most of the phosphatase activity were treated with 0.05% Triton X-100 and chromatographed through DEAE-cellulose. The phosphatase activity elutes from the resin with 0.3 M NaCl. If Triton X-100 is not included in the elution buffer the enzyme binds tightly to the DEAE-cellulose resulting in incomplete recovery, even in the presence of high salt concentrations. The M, 160,000 polypeptide represents more than 90% of the protein in the sample recovered after chromatography on DEAE-cellulose (Fig. 5, lane 3). Other species are visible just below this band but the sizes and amounts of these minor species vary when different alkaline phosphatase preparations are compared. These minor species never represent more than 10% of the total fraction, accumulate in concert with the M, 160,000 polypeptide following removal of phosphate from the medium and react with antibodies prepared against the M, 160,000 species (see below).

**Identification of the Phosphatase.** To confirm the identity of the M, 160,000 polypeptide as the species required for alkaline phosphatase activity, we excised this single polypeptide from a denaturing polyacrylamide gel, electroeluted it, and used it for antibody preparation. Figure 6 shows a Western blot of periplasmic and total soluble proteins screened with these antibodies. The antibodies do not react with periplasmic proteins or with total soluble proteins of phosphate-sufficient cells (lanes 1 and 3) but, as expected, strongly bind to the M, 160,000 polypeptide in both the periplasmic and total soluble fractions from cells deprived of phosphate for 24 h (lanes 2 and 4). Cross-reactivity is also observed with the minor species of the periplasmic fraction which migrate between M, 100,000 and 130,000, confirming that these polypeptides are related to the M, 160,000 polypeptide. However, these lower mol wt polypeptides are not immunologically detectable in the total soluble fraction (lane 4) even when higher levels of protein are electrophoresed (not shown). This suggests that some degradation of the alkaline phosphatase is occurring during the preparation of periplasmic proteins. Other proteins which accumulate in the total soluble fraction of cells deprived of phosphate (e.g. a M, 33,000 polypeptide, see Fig. 6) do not react with the antibodies.

The effect of the antibodies prepared against the M, 160,000 polypeptide on alkaline phosphatase activity in the periplasmic fraction is presented in Figure 7. As a control, we used the IgG fraction from a rabbit producing antiserum against ribulose-1,5-bisphosphate carboxylase. A slight increase in phosphatase activity is measured with high concentration of anti-ribulose-1,5-bisphosphate carboxylase IgG, suggesting that this fraction contains low levels of serum phosphatases. In contrast, antibodies raised against the M, 160,000 polypeptide inhibits the phosphatase activity up to 70%. Furthermore, the antibodies precipitate the enzyme activity in combination with protein A-Sepharose (result not shown). Together the results presented above indicate that the polypeptide responsible for alkaline phosphatase activity which accumulates during phosphate deprivation of _A. nidulans_ is the M, 160,000 polypeptide.

**DISCUSSION**

Experiments presented in this paper confirm the observation that an alkaline phosphatase accumulates in the cyanobacterium _Anacystis nidulans_ during phosphate deprivation (12). This enzyme is sensitive to chelators such as EDTA, a common feature

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**Fig. 4.** Distribution of alkaline phosphatase on a linear sucrose gradient after centrifugation of periplasmic extracts from cells deprived of phosphate for 24 h. Two mg of protein were loaded onto a 10 to 60% sucrose gradient and centrifuged 19 h at 76,000g as described in "Materials and Methods." Protein concentration and phosphatase activity were measured using 150 and 50 μl, respectively, from each 2.4 ml fraction. Phosphatase activity in each fraction is represented by bars and protein concentrations by the solid-lined curve.

**Fig. 5.** SDS-polyacrylamide gel of polypeptides from different stages in the purification of alkaline phosphatase. Lane 1, Periplasmic proteins of cells deprived of phosphate for 24 h; lane 2, proteins of pooled fractions from the sucrose gradient exhibiting the highest phosphatase activity (fractions 3, 4, 5, and 6 of Fig. 4); lane 3, proteins eluted from a DEAE-cellulose column with 0.3 M NaCl exhibiting the highest alkaline phosphatase activity (the fractions with the highest activities from the sucrose gradients were loaded onto the column).
Mg$^{2+}$ is either required for phosphatase activity, or substitutes for another ion bound to the enzyme. Many alkaline phosphatases, including the E. coli enzyme (5, 7), are strongly associated with Zn$^{2+}$. However, Zn$^{2+}$ severely inhibits the A. nidulans enzyme (12) and cannot restore activity after EDTA treatment. The above characteristics of the cyanobacterial enzyme are interesting since they contrast with those of the E. coli enzyme, however, more information is required to define the role of divalent cations in the mechanism of catalysis and the differences between the periplasmic phosphatases of E. coli and A. nidulans.

While several periplasmic polypeptides of A. nidulans disappear following phosphate deprivation, we detect an increase in only a single prominent species having an apparent mol wt of 160,000. Purification of the periplasmic alkaline phosphatase activity, coupled with immunological data, demonstrate that this polypeptide is responsible for the derepressible alkaline phosphatase activity in A. nidulans. The mol wt of this species is larger than that reported for subunits of other alkaline phosphatases, although these values vary over a wide range (from 47,000 for E. coli alkaline phosphatase [2] to 87,000 for bovine kidney alkaline phosphatases [3]). The high mol wt of the A. nidulans monomer raises the possibility that the cyanobacterial enzyme has functions in addition to hydrolyzing phosphate monoesters (e.g. it may play a role in the binding and delivery of phosphate to the transport apparatus on the plasma membrane). We have not yet determined whether the A. nidulans alkaline phosphatase exists as a dimer in vivo, as does the alkaline phosphatase from several other organisms (18).

The release of alkaline phosphatase into the medium upon osmotic shock and the rapid hydrolysis of exogenous p-nitrophenyl phosphate by intact cells indicates that much of the derepressible alkaline phosphatase of A. nidulans, like the analogous enzyme of E. coli (19), is localized in the periplasmic space. Our results on the localization of the alkaline phosphatase of A. nidulans to the periplasmic space are in agreement with previous observations (12); however, using the osmotic shock procedure we observed that only 20 to 30% of the phosphatase activity is released into the medium. These results suggest that the derepressible alkaline phosphatase is extracellular but firmly associates with cytoplasmic membrane or cell wall constituents and is not efficiently released by osmotic shock. This is supported by the observation that a second osmotic shock treatment increases additional activity (MA Block, unpublished data). Furthermore, the A. nidulans phosphatase appears to be associated with either the cell wall or membranes since the majority of the activity is particulate and co-migrates with lipids during sedimentation through a sucrose gradient, and its association with DEAE-cellulose is dramatically altered by the nonionic detergent Triton X-100. Inefficient release of periplasmic phosphatases in Salmonella typhimurium has also been reported (13). In mammalian tissue, some alkaline phosphatases are covalently bound to phosphatidyl inositol (15, 16). In prokaryotes recent work has shown that several proteins are lipoproteins including membrane bound forms of excreted proteins (for a review see Ref. 27). More data are needed to define the interaction of A. nidulans alkaline phosphatase with lipids and/or the bacterial cell wall.

Biochemical and physiological effects of phosphate deprivation have been investigated in both prokaryotic and eukaryotic organisms. In each case the synthesis of alkaline phosphatase correlates with the ability of the organism to survive on phosphate-poor medium. In A. nidulans the cells respond rapidly to phosphate deprivation, and the lag period prior to the detection of increased alkaline phosphatase activity is very short. The periplasmic alkaline phosphatase may be important for acclimation of microorganisms to low phosphate environments by making inorganic phosphate available for transport into the cell by hydrolyzing phosphate ester bonds (releasing phosphate from organic compounds). In addition, phosphate deprivation also re-

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**Fig. 6.** Western blot of periplasmic and total soluble proteins immunostained using antibodies raised to the M, 160,000 polypeptide. Periplasmic proteins (lanes 1 and 2) and total soluble proteins (lanes 3 and 4) of phosphate-sufficient cells (lanes 1 and 3) and cells deprived of phosphate for 24 h (lanes 2 and 4) were resolved by SDS-PAGE and either stained with Coomassie brilliant blue R-250 (A), or transferred to nitrocellulose and screened with antibodies (B). The arrows indicate the major polypeptides which accumulate after 24 h of phosphate deprivation.

**Fig. 7.** Inhibition of phosphatase activity with antibodies prepared against the M, 160,000 polypeptide. The periplasmic fraction (10 μg of protein in 50 μl aliquots) from cells deprived of phosphate for 24 h was incubated for 1 h on ice with increasing amounts of IgG raised against either the M, 160,000 species (□) or Chlamydomonas ribulose-1,5-bisphosphate carboxylase (○). Following the incubation the mixture was assayed for phosphatase activity at 37°C for 5 min.

to most alkaline phosphatases (18). However, following inhibition of the A. nidulans phosphatase with EDTA, most of the activity can be restored by the addition of MgCl₂. Therefore,
sults in elevated levels of phosphate transport by *A. nidulans* (8). When *E. coli* is grown on medium with limiting phosphorus the cells synthesize, in addition to the periplasmic alkaline phosphatase, a variety of different membrane proteins involved in phosphate transport. These proteins, like the alkaline phosphatase, are encoded by genes included in the *pho* regulon (22, 24). Preliminary experiments were conducted in our laboratory to see if alkaline phosphatase and phosphate uptake were stimulated simultaneously in *A. nidulans*. It appears that the phosphate uptake has a longer lag phase of induction (~2 h) than alkaline phosphatase (result not shown). This leads to some questions about the co-regulation of both events in *A. nidulans* and the comparison with *E. coli* regulation. However, phosphate uptake can be modulated by a number of different factors including the induction and utilization of different transport systems, the pH of the local environment around the transport components and the surface potential of the cells (1). Therefore, elevated rates of phosphate uptake during phosphate deprivation must be interpreted cautiously. Cloning of the phosphatase gene is underway in our laboratory and should help us understand its regulated synthesis during phosphate deprivation, and the similarities between regulatory mechanisms in *E. coli* and *A. nidulans*.

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