Isolation and Functional Reconstitution of a Phosphate Binding Protein of the Cyanobacterium Anacystis nidulans Induced during Phosphate-limited Growth*

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Adaptation of the blue-green algae Anacystis nidulans to phosphate-deficient growth leads to the expression of two membrane proteins, which appear as major constituents after separation by gel electrophoresis. One of these proteins, referred to as high affinity phosphate binding protein, has been isolated and its function reconstituted in liposomes. Partial sequencing showed no significant homologies to other proteins. The binding capacity of the proteoliposomes could be inhibited by arsenate but not by sulfhydryl reagents. Scatchard plot analyses of phosphate binding to reconstituted proteoliposomes suggested the existence of two different binding sites, one with a dissociation constant below 30 mM and the other in the micromolar range.

In oligotrophic lakes the phosphate concentration is extremely low and usually does not exceed the nanomolar range (1). In consequence, incorporation of this nutrient has to proceed against a concentration gradient of about 4 or 5 orders of magnitude in Anacystis nidulans (2). The molecular components that catalyze this process have so far not been characterized, and very little is known about the energy-consuming mechanism underlying the translocation of phosphate across the cell membrane. Among other prokaryotes only the phosphate uptake system of Escherichia coli has been characterized in detail at the molecular level (for review see Refs. 3 and 4), including the structural elucidation of the periplasmic binding protein (5). However, since E. coli lives in a completely different environment, where phosphate usually does not become a limiting nutrient, it can be expected that the properties of its uptake system might not be representative for an organism that has to adapt to nutrient fluctuations at or below the nanomolar range.

Using the cyanobacterium A. nidulans we have investigated the membrane proteins that are induced during phosphate-limiting growth conditions. The present report deals with a high affinity phosphate binding protein (HAPBP) that is different from the binding protein in E. coli. It could not be released by cold osmotic shock and revealed a different phosphate binding behavior in reconstitution experiments. Furthermore, the results of partial sequencing of the HAPBP showed no homologies with any characterized protein.

EXPERIMENTAL PROCEDURES

Materials and Growth Conditions—A. nidulans (Synechococcus sp.) Drouet (strain 1402-I, Algal Culture Collection Göttingen) was grown photoautotrophically in medium D of Kratz and Myers (6) at a temperature of 37 °C and supplied with air enriched with 5% CO2. Non-phosphate-deficient algae were cultivated on 3 mM external phosphate in batch cultures. For phosphate-deficient growth algae were cultivated in a 20-liter vessel on 2.5 μM total phosphorus in a discontinuous mode; every 2nd day 10 liters of the suspension was removed for preparation, and the culture was refilled with 10 liters of fresh medium, again containing 2.5 μM KH2PO4, which was incorporated by the starved culture within less than half an hour. The state of deficiency of these algae was monitored by measuring the uptake activity and the threshold value as described previously (7).

Electrophoresis—Analytical gel electrophoresis was carried on 8–22% gradient polyacrylamide minialab gels according to the procedure of Matsudaiera and Burgess (8) in the buffer system of Laemmli (9). Protein samples were supplemented with SDS sample buffer (final concentrations: 2.5% SDS, 1% β-mercaptoethanol, 7% glycerol, 0.001% bromphenol blue, 62.2 mM Tris/HCl, pH 6.8) and boiled for 1 min in a water bath. Gels were stained with Coomassie Brilliant Blue R-250 and destained in 10% acetic acid.

Protein Purification—Cells were harvested by centrifugation at 4,000 × g for 6 min, washed with distilled water by centrifugation, and resuspended in 40 ml of medium I that contained 20% sucrose, 5 mM NaCl, and 10 mM HEPES/KOH, buffered at pH 7.6. EDTA (2 mM) was added to remove the outer membrane, and the cells were incubated at 37 °C with 40 mg of lysozyme to digest the cell walls. After 2.5 h the spheroplasts were centrifuged at 6,000 × g for 6 min, washed with medium II containing 10 mM NaCl, 2.5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 20 mM HEPES/KOH, pH 7.6, and then incubated for 10 min in about 5 ml of the medium II with additional 0.15% Triton X-100 and 300 mM Na2SO4 (or 500 mM Na2SO4 without Triton). The spheroplasts were centrifuged at 20,000 × g for 10 min, and (NH4)2SO4 was added to the supernatant to 3 M final concentration. The precipitated proteins were collected by centrifugation and redissolved in medium II. After further centrifugation at 100,000 × g for 10 min to remove all cell fragments (crude protein extract), 500 μl of the protein suspension was applied to an FPLC Superose 12 column equilibrated in medium II. Proteins were chromatographed at a constant flow rate of 0.3 ml/min−1, and fractions of 1 ml were collected for gel electrophoresis. The phosphate binding activity of individual fractions was assessed in the reconstitution experiments described below.

Protein Blotting and Amino Acid Sequencing—Protein peak fractions from the FPLC column containing the high affinity phosphate binding protein (see Fig. 2) were run on acrylamide minilab gels, and stained bands at around 30 KDa corresponding to the phosphate binding protein were excised with a scalpel and boiled. The gel slices were incubated in Tris/HCl, pH 6.8, three times for 15 min for equilibration and loaded onto a 15% acrylamide gel with 1.5-mm spacers containing a 4% stacking gel constituting about two-thirds of the gel length. The concentrated gel band was electroblotted onto a polyvinylidene difluoride membrane (Immobilon P, Millipore) as described previously (10, 11). The blot was air-dried and stored in aluminum foil at 4 °C.

The excised polyvinylidene difluoride membrane was cleaved in situ with trypsin. Liberated peptides were separated by reversed phase high pressure liquid chromatography on a Bakerbond C18 column in 0.1% trifluoroacetic acid developed with a linear gradient from 0 to 70% acetonitrile.

The abbreviations used are: HAPBP, high affinity phosphate binding protein; FPLC, fast protein liquid chromatography.

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Saccharomyces cerevisiae in water at 30 °C. Peaks were sequenced in an Applied Biosystems 477A automated protein sequenator according to the manufacturer's instructions.

**Reconstitution and Binding Experiments**—The liposomes used for the reconstitution experiments were prepared by the method previously described (12). The lipids employed in this procedure had been extracted from the membranes of *A. nidulans* (13) in order to reconstitute the proteins in a natural lipid environment.

The reconstitution followed a modified freeze-thaw sonication method (12). An aliquot of the protein suspension (50-500 μl, either crude protein extract or purified protein) was added to 50-250 μl of medium, diluted with 1-5 ml of medium described (12). The lipids employed in this procedure had been extracted from the membranes of *A. nidulans* (13) in order to reconstitute the proteins in a natural lipid environment.

The reconstitution was followed by a modified freeze-thaw sonication method (12). An aliquot of the protein suspension (50-500 μl, either crude protein extract or purified protein) was added to 50-250 μl of liposomes that contained 0.05% Triton X-100. The solution was then diluted with 1-5 ml of medium II below the critical micelle concentration of Triton X-100 (0.02% (cf. Ref. 14) and rapidly frozen in liquid nitrogen. After 5 min the sample was thawed in a water bath at room temperature, kept on ice, and sonified twice for 15 s each (W-220 F, Ultrasonics Inc.). The resulting proteoliposomes were centrifuged in an Eppendorf centrifuge, and the pellet was resuspended in medium II and finally analyzed by gel electrophoresis and used for binding experiments.

Protein was determined by the procedure of Bradford (cited in Ref. 15) or following the bicinchoninic acid method (Sigma), which is less disturbed by the presence of detergents.

Binding experiments with the reconstituted system were carried out at room temperature under equilibrium conditions; 5 μl of the respective [32P]phosphate solution was added to 80 μl of proteoliposomes and incubated for about 10 min. For each phosphate concentration double samples were taken.

After incubation the samples were centrifuged, 50 μl of the supernatant and an aliquot of the resuspended pellet was taken, and the radioactivity was measured in water in a scintillation counter.

Arsenate is a competitive inhibitor of phosphate transport (16) that does not enter the cell, as shown by its lack of effect on photophosphorylation in *A. nidulans.* We used this inhibitor to block phosphate binding to proteoliposomes. In the presence of this inhibitor only a small amount of tracer was found in the proteoliposome pellet, independent of whether or not the inhibitor was added before or after the ligand, indicating the absence of phosphate transport into the proteoliposomes (17). Independent experiments with tritium water to determine the pellet water confirmed that the remaining "phosphate binding" in the presence of arsenate corresponded to tracer unspecifically trapped by the centrifuged proteoliposomes. The specific binding was therefore defined as the total binding without arsenate minus the unspecific binding of the proteoliposomes determined in the presence of 5-10 mM Na₂HAsO₄.

The yield of reconstituted proteoliposomes obtained from the starting volume of a 10-20-liter algal culture was too low to allow both binding studies and protein determination. Therefore binding was related to the suspension volume. The dissociation constant obtained in Scatchard plots (18) is, however, unaffected by the reference system.

**RESULTS AND DISCUSSION**

**Changes in Protein Pattern Caused by Phosphate-deficient Growth**—Fig. 1 shows that the synthesis of two proteins in high amount is induced within a few hours after the onset of phosphate-limiting growth conditions (*lanes 1-3, bands I and II*). *Lane 1* shows the proteins of phosphate-unlimited algae. *Lanes 2 and 3* represent the protein pattern in the transition state between static and phosphate-limited growth. The algae applied to *lane 4* were obtained from a culture adapted to permanent phosphate-deficient growth conditions; *lane 5*, protein-free liposomes used for reconstitution; *lane 6*, proteoliposomes reconstituted with crude protein extract (see "Experimental Procedures"). *I*, uncharacterized protein induced by phosphate starvation; *II*, HAPBP. *St*, molecular mass standards in kDa (myosin heavy chain; metavinculin; vinculin; phosphorylase b; bovine serum albumin; pyruvate kinase; hexokinase; actin; carbonic anhydrase; myosin light chain; cytochrome c (from top to bottom)).

![Figure 1](image1.png)

**Fig. 1.** Electrophoretic analysis of the protein pattern of whole cells of *A. nidulans* and of (proteo)liposomes. *Lane 1*, non-phosphate-deficient algae; *lane 2*, batch algae of lane 1, kept in phosphate-free medium for 26 h; *lane 3*, algae of lane 1 for 48 h in phosphate-free medium; *lane 4*, algae adapted to permanent phosphate-deficient growth conditions; *lane 5*, protein-free liposomes used for reconstitution; *lane 6*, proteoliposomes reconstituted with crude protein extract (see "Experimental Procedures"). *I*, uncharacterized protein induced by phosphate starvation; *II*, HAPBP. *St*, molecular mass standards in kDa (myosin heavy chain; metavinculin; vinculin; phosphorylase b; bovine serum albumin; pyruvate kinase; hexokinase; actin; carbonic anhydrase; myosin light chain; cytochrome c (from top to bottom)).

**FIG. 2.** Purification and identification of the HAPBP. Protein concentration of column fractions obtained by gel chromatography of crude phosphate binding activity of the reconstituted proteoliposomes. Binding activity is given as μmol liter⁻¹ and was determined at 1 μM phosphate. *Inset*, SDS-polyacrylamide gel electrophoresis of column fractions as indicated and proteoliposomes reconstituted with fraction 17 (P). *St*, molecular mass standards (200 kDa, myosin; 116 kDa, β-galactosidase; 97.4 kDa, phosphorylase b; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; 31 kDa, carbonic anhydrase; 21.5 kDa, trypsin inhibitor; 14 kDa, lysozyme; 6.5 kDa, aprotinin (from top to bottom)).

![Figure 2](image2.png)

**G. Falkner, unpublished data.**
Nonlinear binding behavior has been resolved into two straight lines of phosphate concentration. The bound and free proportion was determined after centrifugation from the radioactivity in the pellet and supernatant, respectively. Inset, Scatchard plot of the data. The nonlinear binding behavior has been resolved into two straight lines (21) by using the MLAB and Sigma Plot computer programs. The curves of the figure and inset represent the best computer fit. The calculated dissociation constants were 0.29 and 10.3 μM phosphate (S.D. = 0.08 and 0.58, respectively).

Both sequence stretches showed no significant match with any other protein in homology searches in the PIR and SWISS-PROT data base.

Reconstitution—Treatment with 0.1% Triton X-100 and 300 mM Na₂SO₄ did not result in lysis of the spheroplasts. Electrophoretic analysis of the crude protein extract and the remaining membranes showed that this low concentration of detergent removed only the HAPBP quantitatively from the membranes.

This protein solution could be successfully used for reconstitution experiments. Fig. 1, lane 6, shows that only the HAPBP was present in the reconstituted proteoliposomes (the protein band of low molecular weight is due to a contamination with lysozyme, previously used in the preparation of spheroplasts). Although many other proteins were present in the crude extract, apparently none of them associated with the liposomes in significant amount.

Fig. 2 shows the elution profile of the FPLC fractions and the corresponding phosphate binding activity of these fractions reconstituted in proteoliposomes. The fraction that contained the main portion of HAPBP (18 in this experiment; see inset) exhibited the highest activity. Measurements of the binding capacity of fractions flanking the peak showed that the activity specifically followed the presence of HAPBP and was not associated with any of the contaminating proteins in fractions 18 and 19.

Binding Studies with the HAPBP—Binding was investigated using proteoliposomes reconstituted either with the crude protein extract or the purified HAPBP. These preparations showed no activity with or without 300 mM Na₂SO₄. These preparations showed no activity with or without 300 mM Na₂SO₄. These preparations showed no activity with or without 300 mM Na₂SO₄. These preparations showed no activity with or without 300 mM Na₂SO₄.

Some of the proteoliposome preparations did not exhibit the high affinity binding site in the submicromolar range. In these cases the data described a straight line that only reflected the low affinity binding site with dissociation constants between 2 and 10 μM phosphate (cf. Fig. 4, inset, with a dissociation constant of about 4 μM), possibly due to the inactivation of the high affinity binding during preparation. This may indicate that the protein in its native state may have an even higher binding affinity than we were able to measure in the best case.

We suggest that during phosphate-limited growth the synthesis of a phosphate binding protein is induced in A. nidulans. This protein is different from the polypeptide of E. coli in that it shows no sequence homology and seems to be a constituent of the cytoplasmic membrane. The finding of a phosphate binding protein in these algae opens a way to new investigations in the phosphate uptake process.

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Fig. 3. [32P]Phosphate binding to proteoliposomes as a function of phosphate concentration. The curve fit yielded a Kᵣ of 6.5 μM phosphate (S.D. = 0.60). Inset, Scatchard plot of the same data, exhibiting a linear phosphate binding behavior.

Fig. 4. [32P]Phosphate binding to proteoliposomes as a function of phosphate concentration. The curve fit yielded a Kᵣ of 6.5 μM phosphate (S.D. = 0.60). Inset, Scatchard plot of the same data, exhibiting a linear phosphate binding behavior.