Protein phosphorylation in the photosynthetic bacterium *Rhodospirillum rubrum*

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Endogenous protein phosphorylation in cellular fractions from *Rhodospirillum rubrum* was manifested after exposure to [γ-^{32}P]ATP. At least six phosphorylated protein bands of 90, 86, 64, 31, 13 and 11 kDa were found in the cell-free extract. Treatment of the 64-kDa band with V_8 protease yielded smaller radioactive bands. Phosphoserine, phosphothreonine and phosphotyrosine were detected after acid hydrolysis of the phosphorylated fractions. Protein phosphorylation in all the fractions was insensitive to cAMP, did not recognize exogenous protein substrates and was rapidly reverted upon elimination of the excess of [γ-^{32}P]ATP. The chlorophyll-anthena apoprotein from *R. rubrum* chromatophores overlapped the 13-kDa phosphorylated band during gel filtration by high-pressure liquid chromatography suggesting that it is one of the substrates of the protein kinase(s) of *R. rubrum*.

Protein phosphorylation *Protein kinase* *Photosynthetic bacterium* Rhodospirillum rubrum

1. INTRODUCTION

Covalent modification of proteins catalyzed by protein kinases is a ubiquitous motif in biology [1,2]. Regulation of enzymatic activities is the most attractive and most intensely studied biochemical function of protein phosphorylation in eukaryotes [1].

Endogenous protein phosphorylation has recently been reported in prokaryotes [3]. Isocitrate dehydrogenase in *Escherichia coli* [4] and in *Salmonella typhimurium* [5] is regulated by phosphorylation by endogenous protein kinase. Isocitrate dehydrogenase kinase and phosphatase activities in *E. coli* appear to be associated with the same protein and possibly with the same polypeptide chain, the phosphatase activity being strictly dependent on ATP [6]. The protein species and the level of phosphorylation in *E. coli* vary depend on the cellular growth phase [7] suggesting an important role of protein phosphorylation in prokaryote cellular regulation.

A light-modulated, retinal-dependent, reversible protein phosphorylation system has been discovered in *Halobacterium halobium* [8]. The remarkable metabolic flexibility of photosynthetic bacteria capable of phototrophic fermentative and respiratory growth comprises an intricate, yet insufficiently understood regulatory system. Here we show that proteins, in subcellular fractions of the photosynthetic non-sulfur purple bacterium *Rhodospirillum rubrum*, undergo phosphorylation upon exposure to [γ-^{32}P]ATP. One of these proteins seems to be the light-harvesting protein.

2. EXPERIMENTAL

2.1. *Culture conditions and preparation of subcellular fractions*

R. rubrum cells (Van Niel strain S_1) were grown anaerobically in the light as in [9] and sedimented cells were sonicated for 2 min at 4°C in an MSE

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ultrasonic disintegrator Mk 2 in two volumes of a medium containing 20 mM potassium phosphate (pH 7), 5 mM $MgCl_2$, 250 mM sucrose (buffer A) and 0.77 mg of DNase per 10 g cells. The homogenate was centrifuged for 30 min at 20000 $\times$ *g* to remove cell debris and the supernatant solution referred to as cell-free extract, was centrifuged at 105000 $\times$ *g* for 60 min. The resulting supernatant, referred to as soluble fraction, was saved and the sediment containing the intracytoplasmic membrane vesicles, was suspended in 10 volumes of buffer A and centrifuged for 60 min at 105000 $\times$ *g*. Finally, the intracytoplasmic membrane vesicles (suspended in buffer A at 1–2 mg bacteriochlorophyll/ml), the cell-free extract and the soluble fraction were stored at 0°C and used within a few hours of their preparation. Bacteriochlorophyll was determined using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ at 772 nm [10]. Protein was measured by the Lowry procedure [11].

2.2. *Protein phosphorylation in cell fractions*

Cell-free extract (100 μg protein), intracytoplasmic membrane vesicles (20–35 μg bacteriochlorophyll) or soluble fraction (40–70 μg protein) were incubated for 15 min at 30°C in 100 μl of a medium containing 5 mM $MgCl_2$, 20 mM Tris–HCl (pH 8), 10 μg/ml efrapeptin to inhibit the membrane-bound Mg^{2+}–ATPase [12], 60 μM vanadate and 0.1 mM ATP containing 0.2 μCi [γ-^{32}P]ATP. The assays were terminated by addition of 70 μl gel electrophoresis sample buffer followed by incubation at 37°C for 1 h.

2.3. *Gel electrophoresis and autoradiography*

Samples were electrophoresed in 15% polyacrylamide slab gel containing SDS, fixed, stained with Coomassie blue and destained as described [13]. Destained gels were dried and exposed for 36 h at −20°C to a Kodak X-ray film with a Kyokko HS intensifying screen.

2.4. *Phosphoamino acid analysis*

Treatment of the samples after phosphorylation and phosphoamino acid analysis by high voltage paper electrophoresis in one [14] or two successive dimensions [15] were carried out as described. Phosphoamino acids were detected with ninhydrin and radioactive spots were detected by autoradiography as described above. The radioactive spots were cut and counted in 3 ml scintillation cocktail (0.7% 2,5-diphenyloxazole and 30% Triton X-100 in toluene) in a Beckman 8100 scintillation counter. The relative ratio of each phosphoamino acid in a given sample was calculated as the ratio between the radioactivity incorporated in a particular phosphoamino acid divided by the radioactivity incorporated in all the phosphoamino acids. Samples were analyzed side by side when their relative ratio of phosphoamino acids was compared.

2.5. *Chemicals*

Phosphoserine, phosphothreonine, phosvitin, histone IIA, histone IIIA, cyclic AMP and DNase were from Sigma Chemical Co. M_r markers for SDS-electrophoresis were from Bio-Rad. *Staphylococcus aureus* V_8 protease was from Miles Laboratories. Carrier-free $^{32}P_i$ was from Comisión Nacional de Energía Atómica, Argentina. Salt-free, carrier-free [γ-^{32}P]ATP was prepared as in [18]. Phosphotyrosine was a generous gift from Dr Tony Hunter, The Salk Institute, San Diego, CA, USA. Other chemicals used were reagent grade.

3. RESULTS

Incubation of a cell-free extract from *R. rubrum* cells with [γ-^{32}P]ATP revealed at least 6 phosphorylated protein bands of 90, 86, 64, 31, 13 and 11 kDa, respectively (fig.1, lanes 1 and 2). Resolution of the cell-free extract into soluble fraction and intracytoplasmic membrane vesicles and subsequent exposure of the fractions to [γ-^{32}P]ATP rendered three phosphorylated bands of 90, 64 and 31 kDa in the soluble fraction and two phosphorylated bands of 86 and 11 kDa in the membrane fraction (fig.1, lanes 4 and 6). The 13-kDa phosphorylated band (fig.1, lane 2, e) of the extract did not show in either of the resolved fractions. Phosphorylation of this band may be particularly labile or alternatively, an appropriate recombination of the fractions may be required to restore its phosphorylation.

With the exception of the 11-kDa band only, all the phosphorylated bands in the fractions overlapped the bands stained by Coomassie blue (fig.1). Furthermore, phosphoamino acid analysis of the phosphorylated cell-free extract indicates

the presence of phosphoserine, phosphothreonine and phosphotyrosine in a relative ratio of 0.36, 0.25 and 0.38, respectively. The relative ratio of phosphoserine, phosphothreonine and phosphotyrosine was 0.32, 0.16 and 0.52 in the phosphorylated membrane vesicles and 0.66, 0.25 and 0.09 in the phosphorylated soluble fraction.

Upon elimination of the excess of [γ-^{32}P]ATP the phosphorylated soluble fraction and intracytoplasmic membrane vesicles rapidly lost their radioactivity (fig.2). Dephosphorylation in both fractions was insensitive to 20 mM NaF (not shown). The addition of 5 mM EDTA to the medium prevented dephosphorylation of the membrane fraction (fig.1A) but had no effect on the dephosphorylation of the soluble fraction (fig.2B).

Phosphorylation of the fractions was insensitive to 10 μM cAMP and did not recognize exogenous protein substrates such as casein, histones or phosvitin.

Ageing of the fractions under different conditions impairs the phosphorylation. For instance, when the soluble fraction was stored for several hours on ice before exposure to [γ-^{32}P]ATP, phosphorylation of the 90- and 31-kDa bands was not observed while phosphorylation of the 64-kDa band was enhanced (fig.3, lane 2). Treatment of the 64-kDa band with V_8 protease rendered 4 radioactive bands of 30, 28, 18 and 16 kDa (fig.3, lane 3). Moreover, when the cell-free extract was frozen under liquid nitrogen and thawed at room temperature before exposure to [γ-^{32}P]ATP, a

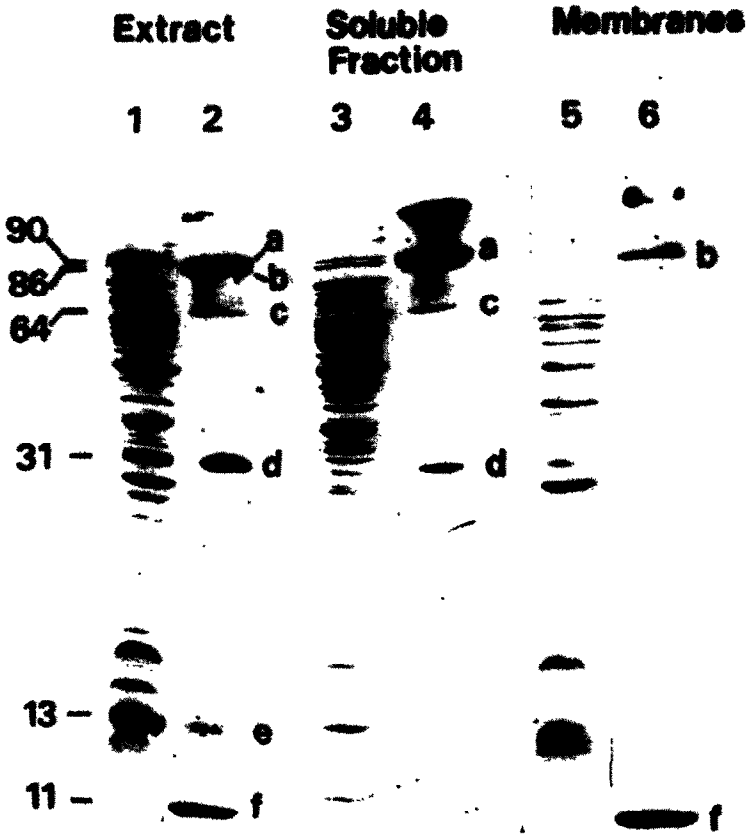

Fig.1. Phosphorylation of the cell-free extract and resolved fractions from *Rhodospirillum rubrum*. Phosphorylation of the fractions with [γ-^{32}P]ATP was carried out as described in the text. Odd numbered lanes are the Coomassie blue-stained, and even numbered lanes are their corresponding autoradiograms. Letters at the right-hand side of lanes 2, 4 and 6 indicate the phosphorylated bands. Figures at the left-hand side identify their molecular masses, e.g., 90 = 90 kDa.

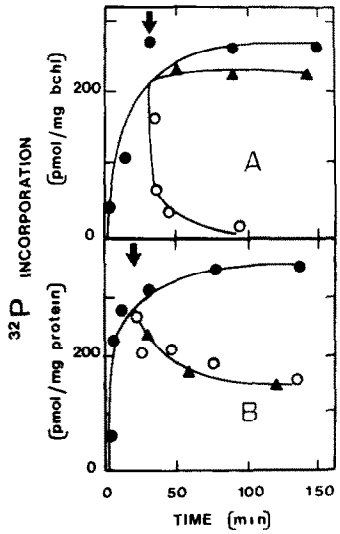

Fig.2. Dephosphorylation of the intracytoplasmic membrane vesicles and soluble fraction. (A) Time course of phosphorylation (●—●), dephosphorylation (○—○) and dephosphorylation in the presence of 5 mM EDTA (▲—▲) of the intracytoplasmic membrane vesicles carried out as described in the text. (B) As in A, but for the soluble fraction. Arrows indicate the time at which samples (20 μl) were quickly subjected to centrifugation–filtration [17] to eliminate the excess of [γ-^{32}P]ATP. ^{32}P incorporated into the proteins was determined as in [16].

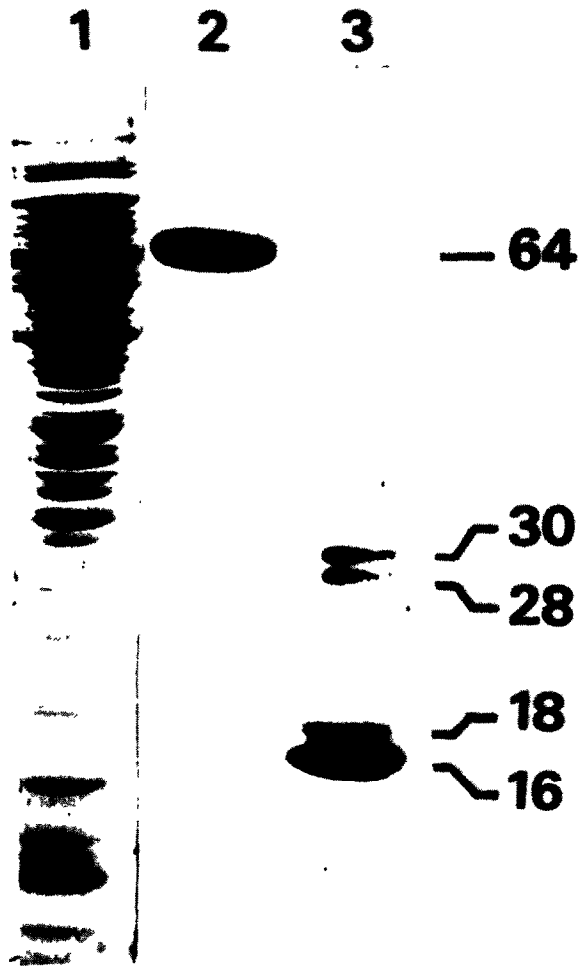

Fig.3. Proteolytic cleavage of the phosphorylated soluble fraction. The soluble fraction was left on ice for 5 h and then phosphorylated with [γ-^{32}P]ATP as described in the text. The excess of [γ-^{32}P]ATP was eliminated as described in the legend to fig.2 and a fraction (0.5 mg protein/ml) was incubated with 10 μg/ml V_8 protease at 27°C for 8 h. Lane 1, Coomassie blue-stained soluble fraction; lane 2, autoradiogram; lane 3, autoradiogram after V_8 protease treatment.

lower level of phosphorylation was observed (fig.4A,B), particularly in the low molecular mass region. Interestingly, the purified apoprotein of the light-harvesting chlorophyll–protein complex from *Rhodospirillum rubrum* chromatophores overlapped the 13-kDa phosphorylated band (fig.4C).

4. DISCUSSION

Based on the overlapping of the phosphorylated bands with Coomassie blue-stained bands (fig.1) the sensitivity to proteolytic treatment of the 64-kDa band (fig.3) and the phosphoamino acid detected, we conclude that protein phosphorylation does occur in subcellular fractions from *R. rubrum* cells. Protein phosphorylation was also observed in the resolved fractions from cell-free extracts (fig.1) suggesting that the protein kinases and their corresponding substrates distribute between membranes and cytoplasm in *R. rubrum* cells. Dephosphorylating activities also distribute in the resolved fractions (fig.4) suggesting the presence of phosphatases. A major drawback in the purification of protein kinases from prokaryote organisms is the lack of exogenous protein substrates to trace the activity [3]. Therefore, the progress in the characterization of prokaryote protein kinases has necessarily involved the purifica-

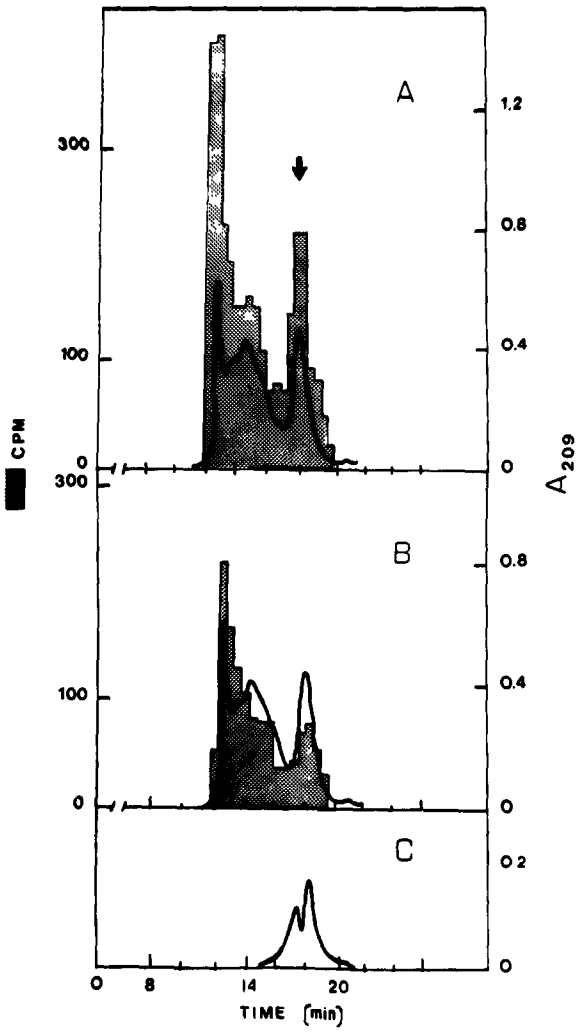

Fig.4. High-pressure liquid chromatography of the phosphorylated cell-free extract. Cell-free extract was phosphorylated as described in the text and the reaction was terminated by addition of SDS at a final concentration of 2%. Freshly prepared (A), frozen-thawed (B) cell-free extract (150 μg protein) or (C) purified light-harvesting apoprotein (40 μg protein) were injected to a TSK 6,300 (Toyo Soda Ltd.) column which was equilibrated with 0.3% SDS and 0.2 M NaCl and the column was developed with the same solution at a flow rate of 1 ml/min using a Waters M 6000A solvent delivery system. The absorbance of the effluent (——) was monitored at 209 nm in a Gilson Holochrome spectrophotometer and 0.4-ml fractions were collected and counted for radioactivity (shaded areas).

tion of the endogenous substrates undergoing phosphorylation [3,4,7]. However, utilizing synthetic peptides, we have recently succeeded in detecting a tyrosine protein kinase associated to the soluble fraction (submitted).

Our observation that the purified apoprotein from the light-harvesting complex overlaps the 13-kDa phosphorylated band strongly suggests that the anthena in *R. rubrum* could be subjected to phosphorylation–dephosphorylation cycles under certain conditions. A recent communication reports that the apoprotein increases its phosphate content under conditions supporting cooperativity among photosynthetic units, that is in the light and in the presence of Mg^{2+} [19].

Further studies are being carried out to characterize the substrates, the kinases and the probable involvement of these activities in the remarkable adaptative metabolism of *R. rubrum*.

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