

Na⁺-ATPase from the plasma membrane of the marine alga *Tetraselmis (Platymonas) viridis* forms a phosphorylated intermediate

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Abstract Plasma membranes isolated from the marine unicellular alga *Tetraselmis (Platymonas) viridis* were phosphorylated by [γ -³²P]ATP, and membrane proteins were then analyzed by PAGE in SDS, under acidic conditions. Three radioactive components with apparent molecular masses of 100 kDa, 76 kDa, and 26 kDa were detected. The phosphorylation of one of them, the 100 kDa polypeptide, was specifically stimulated by Na⁺. Vanadate almost completely inhibited the Na⁺-mediated phosphorylation of the peptide. The phosphate bound to this peptide underwent rapid turnover and was discharged by hydroxylamine. The 100 kDa phosphopeptide was sensitive to ADP. The conclusion is drawn that the 100 kDa phosphopeptide is a phosphorylated intermediate of the Na⁺-transporting ATPase in the *T. viridis* plasma membrane.

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Key words: Plasma membrane; Phosphointermediate; Sodium pump; Sodium ATPase; *Tetraselmis viridis*; *Platymonas viridis*

1. Introduction

The ion-transporting E₁E₂-ATPases from various organisms, both prokaryotic and eukaryotic, possess the common feature of forming phosphorylated intermediates in the course of the catalytic cycle [1]. The family of the ATPases being phosphorylated during ATP hydrolysis comprises animal Na⁺,K⁺-ATPase [2–4], the sarcoplasmic reticulum Ca²⁺-ATPase [5], the K⁺-ATPase of *Escherichia coli* [6], the H⁺-ATPase of higher plants [7] and yeasts [8–10]. Belonging to the family of E₁E₂-ATPases is indicated by sensitivity to orthovanadate, the inhibitory effect of which is mediated by its ability to bind to the E₂ form of the enzyme [11].

A primary Na⁺-pump has recently been demonstrated to operate in the plasma membrane (PM) of the marine unicellular alga *Tetraselmis (Platymonas) viridis* [12,13]. ATP-driven ²²Na⁺ accumulation by the PM vesicles isolated from this alga occurred in the presence of the protonophore *m*-chlorocarbonylcyanide phenylhydrazone (CCCP), thus indicating a

proton motive force (pmf)-independent fashion of Na⁺-pumping [12]. The ²²Na⁺ accumulation occurred in a weakly alkaline region with a maximum at pH 7.8–8.0 and required penetrable anions in the medium, which argues in favor of electrogenicity of the Na⁺-pump [13]. The ATP-induced Na⁺ translocation across the vesicle membrane [13] as well as ATP hydrolysis catalyzed by the PM preparations from *T. viridis* [14] were sensitive to orthovanadate, suggesting the primary Na⁺-pump of *T. viridis* is an E₁E₂-ATPase, which forms a phosphointermediate in the course of its catalytic cycle.

In the work presented here, we report the findings of phosphorylated proteins in PM preparations isolated from *T. viridis*, the phosphoproteins being formed when the PM fractions were incubated with [γ -³²P]ATP. The obtained data provide evidence that the 100 kDa phosphoprotein in the PM of *T. viridis* formed specifically in the presence of Na⁺ ions is a phosphointermediate of the Na⁺-transporting ATPase.

2. Materials and methods

T. viridis cells were cultured in artificial sea water at 0.46 M NaCl [13]. The highly purified PM fractions were isolated as described previously [14]. The final PM pellets were suspended in medium containing 0.5 M glycerol, 10 mM HEPES-BTP buffer, pH 7.2, 1 mM DTT, 1 mM PMSF, and 0.2 mM EGTA.

Phosphorylation reactions were carried out according to the method of Post and Sen [15] at ice temperature in a 0.2 ml reaction volume containing 30 mM BTP-HEPES, pH 8.0, 0.1 mM MgCl₂, 25 mM NaCl, 15 nM [γ -³²P]ATP (3000 Ci/mmol), and 20–25 µg of membrane protein. This composition of the reaction mixture is referred to as the standard reaction mixture. The reaction was started by the addition of [γ -³²P]ATP and quenched by the addition of 1 ml of ice-cold 10% TCA containing 40 mM NaH₂PO₄, 5 mM Na₄P₂O₇ and 1 mM ATP (disodium salt). The quenched reaction mixture was centrifuged at 100 000 × *g* for 15 min. The primary TCA pellets were washed twice by centrifugation at 10 000 × *g* for 15 min in 1 ml of the quenching solution.

The effect of hydroxylamine was tested by incubating the TCA pellets in 50 mM MES-BTP, pH 5.2, in the presence or absence of 0.25 mM hydroxylamine for 30 min on ice.

Samples for acidic electrophoresis were prepared by dissolving final precipitates in 50 µl of a solution containing 10 mM (H₃+NaH₂)PO₄, pH 2.4, 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, 4 M urea, 20% glycerol, and 10 µg/ml pyronin Y. The samples were incubated for 30 min at ice temperature and applied to the gel.

The 5.6% polyacrylamide gels at pH 2.4 containing 0.1% SDS were prepared according to Fairbanks and Avruch [16]. The electrophoresis was performed at a constant current of 40 mA and 5°C. The running times for 70 mm tracking dye migration were 2.5–3.0 h.

After acidic electrophoresis the gels were immediately dried under vacuum and subjected to autoradiography (1–3 h at –80°C) using Retina X-ray film with intensifying screen. The molecular masses of the phosphorylated bands were estimated by comparison with Sigma prestained mass markers (27 000–180 000 Da) run in adjacent lanes.

Protein content was determined by the micromethod of Simpson and Sonne [17] with bovine serum albumin as a standard.

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Abbreviations: DTT, dithiothreitol; CCCP, *m*-chlorocarbonylcyanide phenylhydrazone; PM, plasma membrane; pmf, proton motive force; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid

3. Results

3.1. Pattern of protein phosphorylation

When the PM fractions isolated from *T. viridis* were incubated with [γ - 32 P]ATP in the presence of 25 mM Na⁺ and 0.1 mM Mg²⁺, and subsequently the PM proteins were analyzed by acidic SDS-PAGE, three bands corresponding to 100 kDa, 76 kDa, and 26 kDa polypeptides were observed in the autoradiographs (Fig. 1, lane 1).

The treatment of the samples with hydroxylamine after the reaction was stopped resulted in elimination of all these bands (Fig. 1, lane 2). The sensitivity to hydroxylamine suggests acyl-phosphate bonds forming in the protein in the course of the reaction.

3.2. Ionic relations

Phosphorylation of the 100 kDa protein required specifically the presence of Na⁺ in the reaction medium; when Na⁺ was omitted from the standard reaction mixture, the 100 kDa band was absent from the autoradiographs (Fig. 1, lane 3 vs. 1). In this reaction Na⁺ could not be replaced by other monovalent cations such as K⁺, Li⁺, NH₄⁺, and Cs⁺ (data not shown). At the same time, Mg²⁺ was not necessary for phosphorylation of the 100 kDa protein. Na⁺-mediated 100 kDa protein phosphorylation occurred even more efficiently in the absence of Mg²⁺ (Fig. 1, lane 5 vs. 1) while increasing Mg²⁺ concentrations up to 5 mM led to a considerable reduction in the 100 kDa band (Fig. 1, lane 7 vs. 1 and 5). In pulse-chase experiments, 5 mM MgCl₂ added after 30 s phosphorylation caused dephosphorylation of the 100 kDa protein (Fig. 2, lane 2 vs. 1).

The 76 kDa protein did not require Na⁺ to be phosphorylated (Fig. 1, lane 3) but required the presence of Mg²⁺. Labeling of the 76 kDa protein was not observed in the absence of Mg²⁺ (Fig. 1, lane 5 vs. 3); increasing Mg²⁺ concentrations

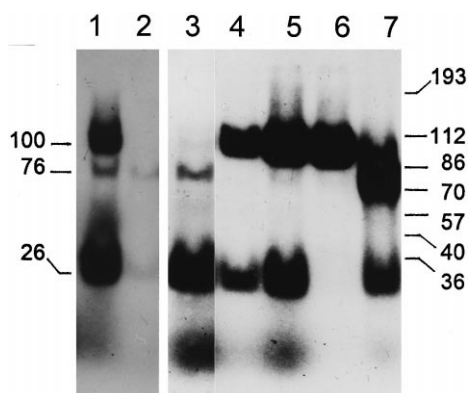


Fig. 1. Characteristics of phosphoprotein formation in the PM preparations from *T. viridis*. Phosphorylation for 30 s, hydroxylamine treatment and electrophoresis were carried out as described in Section 2. Lane 1, the standard reaction mixture, buffer control to hydroxylamine treatment; lane 2, hydroxylamine treatment; lane 3, the standard reaction mixture lacking sodium; lane 4, the standard reaction mixture, sodium concentration enhanced up to 100 mM; lane 5, the standard reaction mixture lacking magnesium; lane 6, 30 s phosphorylation in the standard reaction mixture lacking magnesium followed by a 10 s chase with 5 mM unlabeled ATP; lane 7, the standard reaction mixture, magnesium concentration enhanced up to 5 mM.

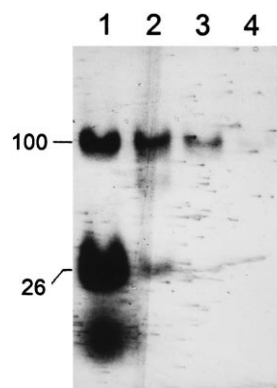


Fig. 2. Chases with magnesium, ATP, and ADP. The phosphorylation reaction was allowed to proceed for 30 s in the standard reaction mixture lacking magnesium, and then additions were made for 10 s. Lane 1, no addition; lane 2, 5 mM MgCl₂; lane 3, 5 mM MgCl₂+5 mM ATP cold; lane 4, 5 mM ADP.

up to 5 mM led to enhancement of the 76 kDa band (Fig. 1, lane 7 vs. 1 and 3).

Apparently, no ions are needed for phosphorylation of the 26 kDa protein, since exclusion of Na⁺ or Mg²⁺ from the standard reaction mixture had no visible effect on labeling of the 26 kDa protein (Fig. 1, lanes 3, 5). In contrast, increasing the Na⁺ or Mg²⁺ concentrations led to decreased label incorporation into this protein (Fig. 1, lanes 4 and 7 vs. 1). In pulse-chase experiments, 5 mM MgCl₂ caused rapid dephosphorylation of the 26 kDa protein (Fig. 2, lane 2 vs. 1).

3.3. Effects of ATP and ADP

In pulse-chase experiments, an excess of unlabeled ATP added together with Mg²⁺ 30 s after the reaction started caused a marked elimination of the label initially incorporated into the 100 kDa polypeptide and a complete dephosphorylation of the 26 kDa polypeptide (Fig. 2, lane 3 vs. 1). In the absence of Mg²⁺, ATP chase led to rapid discharge of the 26 kDa phosphoprotein as well. The 100 kDa phosphoprotein

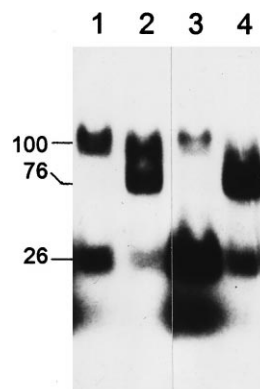


Fig. 3. Effect of orthovanadate on phosphoprotein formation in the PM preparations from *T. viridis*. Phosphorylation reactions were carried out for 60 s. Lane 1, the standard reaction mixture lacking magnesium; lane 2, the standard reaction mixture with enhanced magnesium concentration up to 5 mM; lane 3, the standard reaction mixture lacking magnesium+0.5 mM vanadate; lane 4, the standard reaction mixture with enhanced magnesium concentration up to 5 mM+0.5 mM vanadate.

was not dephosphorylated under these conditions (Fig. 1, lane 6 vs. 5).

5 mM ADP applied in the pulse-chase experiments completely released the label incorporated into 100 kDa and 26 kDa polypeptides (Fig. 2, lane 4 vs. 1).

3.4. Effect of orthovanadate

Orthovanadate significantly reduced the label incorporation into the 100 kDa polypeptide and enhanced labeling of the 26 kDa polypeptide. Labeling of the 76 kDa protein did not practically respond to the addition of orthovanadate (Fig. 3).

4. Discussion

Three polypeptides, of 100 kDa, 76 kDa, and 26 kDa, binding inorganic phosphate into the acyl-phosphate group, were found in the PM fraction of *T. viridis* when the phosphorylation reaction was conducted with γ - ^{32}P -labeled ATP substrate (Fig. 1). One of them, the 100 kDa polypeptide, specifically required Na^+ to be phosphorylated.

The 100 kDa phosphorylated polypeptide from *T. viridis* exhibits molecular properties characteristic of transport ATPases. The observed effects of Mg^{2+} , ATP and ADP on the 100 kDa phosphoprotein formation are consistent with the properties exhibited by the phosphointermediates of E_1E_2 -type ATPases. According to the Albers-Post scheme for animal Na^+, K^+ -ATPase [18], covalent binding of the phosphate by the enzyme in the course of the catalytic cycle (formation of E_1P) is accompanied by Na^+ occlusion into the high-energy phosphoenzyme form, $\text{E}_1\text{P}(\text{Na}^+)$, sensitive to ADP. This reaction does not occur in the absence of Na^+ . The following stage, the conversion of the $\text{E}_1\text{P}(\text{Na}^+)$ into the E_2P and release of Na^+ , requires Mg^{2+} . Mg^{2+} and ATP at high concentrations promote turning over the cycle. At low ATP concentrations in the presence of Na^+ and in the absence of Mg^{2+} , the enzyme is preferentially present in the $\text{E}_1\text{P}(\text{Na}^+)$ form. A high level of the 100 kDa protein labeled by phosphate was observed under these conditions in our experiments (Fig. 1).

The sensitivity of the formed 100 kDa phosphoprotein to ADP (Fig. 2) indicates most likely the presence of the high energy phosphorylated intermediate of *T. viridis* ATPase in the $\text{E}_1\text{P}(\text{Na}^+)$ form that reacts with ADP resynthesizing ATP.

The decrease in labeling of the 100 kDa protein as Mg^{2+} concentrations rise, as well as the rapid dephosphorylation of the 100 kDa phosphopeptide under high Mg^{2+} concentrations in the pulse-chase experiments, indicates that Mg^{2+} stimulates the breakdown of the phosphoprotein rather than inhibiting its formation promoting the conversion of the $\text{E}_1\text{P}(\text{Na}^+)$ into E_2P . The latter then turns into the non-phosphorylated E_2 form.

The discharge of the 100 kDa phosphoprotein in the presence of unlabeled ATP given together with Mg^{2+} indicates that the phosphoprotein undergoes a rapid turnover under these conditions.

The ability to bind terminal phosphate from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the form of an acyl-phosphate group, the specific requirement of Na^+ for phosphorylation, the sensitivity of the reaction to vanadate, and similarities in molecular properties with the E_1E_2 -type ATPases from other organisms indicate that the 100 kDa protein in the PM of *T. viridis* is a catalytic unit of a Na^+ -transporting ATPase. The molecular mass of 100 kDa corresponds to molecular masses of major catalytic units

(subunits) of a number of E_1E_2 -ATPases, which are also 100 kDa polypeptides, being phosphorylated during the catalytic cycle [1–10].

The Na^+ -ATPase forming a phosphointermediate has been revealed recently in the PM of another marine alga, *Heterosigma akashiwo* [11]. It should be noted that the Na^+ -ATPase from *H. akashiwo* differs in some respects from the *T. viridis* enzyme. The former is a 150 kDa polypeptide requiring Mg^{2+} together with Na^+ to be phosphorylated. K^+ ions led to dephosphorylation of the phosphoenzyme from *H. akashiwo* whereas the phosphoenzyme from *T. viridis* was unaffected by K^+ (data not shown).

The functional role of the 76 kDa and 26 kDa polypeptides in PM of *T. viridis* remains obscure.

Some reasons permit us to relate the 76 kDa protein to the H^+ -ATPase in the PM of *T. viridis*. Earlier, ATP-dependent H^+ -pumping into PM vesicles isolated from *T. viridis* was demonstrated by us [19].

The requirement of Mg^{2+} for the phosphorylation of the 76 kDa polypeptide is consistent with an important role of Mg^{2+} in the formation of the phosphointermediate of plant PM H^+ -translocating ATPase [7]. The sensitivity of the 76 kDa phosphoprotein to hydroxylamine indicating an acyl-phosphate bond is also a common feature of the transport ATPase intermediates. Although the level of the 76 kDa phosphopeptide was not reduced by the ATPase inhibitor vanadate, this finding itself cannot be a strong argument against the possibility that the 76 kDa protein is a catalytic unit of H^+ -ATPase. A similar observation has been made for the H^+ -translocating ATPase from the yeast PM, where vanadate did not affect the phosphorylation of the enzyme [20].

The 26 kDa phosphoprotein has some characteristics of the E_1E_2 -ATPase intermediate, namely, sensitivity to hydroxylamine and rapid turnover. High concentrations of Na^+ or Mg^{2+} , which led to an enhancement of the formation of the 100 kDa or 76 kDa phosphoproteins, respectively, caused a decrease in labeling of the 26 kDa protein (Fig. 1). Vanadate, which inhibited labeling of the 100 kDa protein, enhanced labeling of the 26 kDa protein (Fig. 3). This fact may reflect the inhibition by vanadate of phosphate transfer from the 26 kDa protein to the 100 kDa protein. Altogether, these findings suggest that the 26 kDa polypeptide participates in ATP hydrolysis being a site of primary phosphate binding, with subsequent transfer of the phosphate to the 100 kDa or 76 kDa polypeptides. Obviously, that is a point which requires further studying.

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