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Phosvitin kinase activity in *Acholeplasma axanthum*

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Incubating the soluble fraction derived from *A. axanthum* with phosvitin and [γ-^{32}P]ATP results in the phosphorylation of phosvitin. Casein, histone and kemptide were practically ineffective substrates, whereas a 55 kDa protein of *M. gallisepticum* was efficiently phosphorylated. The enzymatic activity has an optimal pH in the pH range 6.0–6.2 and requires divalent cations. The activity was inhibited by ammonium sulfate, heparin and sulphydryl blocking reagents, but was not affected by calmodulin with or without Ca^{2+} or by cyclic AMP.

Phosvitin kinase; Phosphorylation; Enzyme activity; Inhibition

1. INTRODUCTION

The reversible phosphorylation of proteins has been shown to be an established mechanism by which intracellular events in eukaryotes are controlled [1,2]. During the last decade, a growing body of evidence has accumulated regarding the presence of protein kinase activity, as well as the respective endogenous protein substrates in bacteria [3]. Recently, the existence of a protein kinase, a 55 kDa endogenous protein that serves as the major endogenous substrate and a phosphoprotein phosphatase capable of dephosphorylating the endogenous 55 kDa phosphoprotein was demonstrated in *Mycoplasma gallisepticum* [4]. The reversible phosphorylation process was found in several flask-shaped *Mycoplasma* species as well, but not in the *Acholeplasma* species (Platt, M.W., unpublished). The data presented in this study demonstrate and partially characterize a protein kinase activity in *Acholeplasma axanthum*. The kinase is capable of phosphorylating phosvitin, as well as the 55 kDa protein of *M. gallisepticum*. This finding may open the way for the characterization of the reversible phosphorylation of the 55 kDa protein of the flask-shaped mycoplasmas.

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2. MATERIALS AND METHODS

2.1. *Organism, growth conditions and the isolation of cell fractions*

Acholeplasma axanthum and *Mycoplasma gallisepticum* were grown at 37°C in a modified Edward medium [5] supplemented with 4% horse serum. The cells were harvested at the mid-exponential phase of growth by centrifugation at 11000 × *g* for 10 min. The cells were washed twice and resuspended in a solution containing 0.25 M NaCl in 20 mM Tris-HCl buffer, pH 7.4. *A. axanthum* cells were osmotically lysed after preloading with glycerol as previously described [5]. Membranes were separated from the soluble fraction by centrifugation at 37000 × *g* for 30 min and the soluble fraction was concentrated to 20 mg protein/ml by freeze drying, centrifuged at 100000 × *g* for 2 h to remove residual membrane fragments and stored at −70°C until used. *M. gallisepticum* cell extracts were prepared as previously described [4]. Protein in the fractions was determined by the method of Bradford [6].

2.2. *Phosvitin kinase activity*

Phosvitin kinase was assayed in a reaction mixture (final volume of 50 μl) containing 20 mM Tris-maleate buffer (pH 6.0), 1 mM dithiothreitol, 50 mM NaF, 0.2 mM $MgCl_2$, 20–100 μg phosvitin, 20 μg of cell protein and 1 mM of [γ-^{32}P]ATP (100 Ci/mol, The Radiochemical Centre, Amersham, England). The phosvitin preparations utilized (Sigma) were dissolved in 0.1 M citric acid (25 mg/ml) and heated at

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80°C for 15 min. The pH of the phosvitin solution was then adjusted to pH 6.0 with NaOH and the phosvitin was extensively dialyzed and kept at −20°C until used. In some experiments, phosvitin was replaced by (25–100 μg) histone, kemptide or casein (all products of Sigma), or by 50 μg of heat inactivated (80°C, 10 min) *M. gallisepticum* cell extract. The reaction was carried out for 1–2 min at 37°C and was terminated by the addition of 50 μl of denaturing SDS buffer (200 mM Tris-HCl, pH 6.8, 6% SDS, 1% 2-mercaptoethanol, 4% glycerol and 0.005% bromophenol blue) and immediate boiling for 3 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [7] utilizing 10% acrylamide gels. The gels were stained with Coomassie brilliant blue or with Stains All [8] at 50°C and destained for 5 h in methanol/acetic acid/water (15.5:2.5:10, by vol.). The gels were then dried and exposed to X-Omat (Kodak) at −70°C for autoradiography. Radioactivity in the polypeptide bands was quantified by excising the appropriate stained bands and counting them in scintillation liquid. For quantitative distribution of radioactivity in phosvitin and ATP, the phosphorylated phosvitin was separated from excess of ATP and P_i by descending paper chromatography on DEAE-Cellulose (Whatman) using 0.22 M ammonium formate as the solvent [9].

3. RESULTS AND DISCUSSION

3.1. *Labelling of phosvitin by [γ-^{32}P]ATP*

The failures to demonstrate protein kinase activity in prokaryotes were attributed to the use of exogenous substrates of eukaryotic origin for the kinase reaction [3]. Recently, a protein kinase was described in *M. gallisepticum* and it was shown that, in addition to the 55 kDa endogenous protein phosphorylated, the kinase is capable of phosphorylating phosvitin [4]. Fig.1B shows that by incubating the soluble fraction of *A. axanthum* with [γ-^{32}P]ATP and phosvitin, ^{32}P was incorporated to a large extent into phosvitin. The phosvitin preparation contained a cluster of phosphoprotein (phosvettes) dominated by 28, 36 and 43 kDa bands [8]. All three major phosvettes were radiolabelled and the labelling intensities were in accordance with their relative abundance in the phosvitin preparation utilized in the reaction mixture. The radioactive phosvettes were detected after short autoradiography exposures (10–20 h). When phosvitin was omitted from the reaction mixture, no radiolabelled protein bands were detected, even after long autoradiography exposure (7 days, fig.1A), indicating the absence of an endogenous phosphorylated protein substrate in this organism. Phosvitin could not be replaced in the reaction mixture by histone, kemptide or casein (not shown), but when replaced by a heat inactivated *M. gallisepticum* cell extract, a phosphorylated 55 kDa protein was detected (fig.1C). The phosphorylation of phosvitin was almost the same with the soluble fraction obtained from *A. axanthum* cells harvested at the early (14–16 h of incubation, A_{640} = 0.10–0.15) or late (18–20 h, A_{640} = 0.22–0.32) exponential phases of

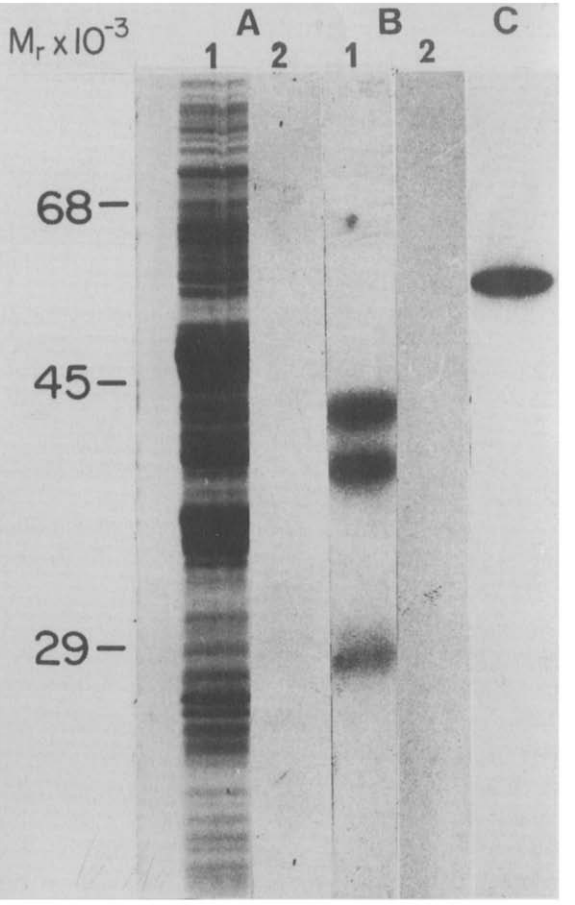

Fig.1. In vitro phosphorylation of phosvitin and of a 55 kDa mycoplasma protein by *A. axanthum*. Phosphorylation was carried out as described in section 2 in a reaction mixture containing *A. axanthum* soluble fraction and [γ-^{32}P]ATP with or without an exogenous protein substrate. (A) Reaction mixture without an exogenous substrate. Coomassie blue-stained gel (1), and an autoradiogram of (1) after 7 days of exposure (2). (B) Reaction mixtures with phosvitin (100 μg). Autoradiograms of gels after 12 h exposure obtained from a reaction mixture containing native (1) or heat-inactivated (80°C, 10 min) *A. axanthum* soluble fraction (2). (C) Autoradiogram (24 h exposure) of a reaction mixture with heat-inactivated *M. gallisepticum* cell extract (50 μg protein).

growth. Much lower activity was found in soluble fractions from stationary phase cells (>26 h of incubation). Under identical conditions, no phosvitin kinase activity was detected in *A. laidlawii* or *A. granularum*.

The phosphorylation of phosvitin is interpreted by us as signifying that *A. axanthum* possesses a phosvitin kinase activity. This interpretation is based on the following findings: (i) no phosphorylation was obtained when the cell free extract was heat denatured (80°C, 10 min, fig.1A) ruling out a nonenzymatic radiolabelling; (ii) phosvitin phosphorylation was obtained with [γ-^{32}P]ATP, but not with [α-^{32}P]ATP, ruling out a ^{32}P-labelled nucleotide binding [10]; (iii) the phosphorylated phosvitin is acid resistant (determined by soaking acrylamide gel slices in 1 M HCl for 1 h at 60°C prior to autoradiography), ruling out the possibility of a phosphoamidate linkage [11]; (iv) the phosphorylated phosvitin is base labile consistent with a phosphomonoester of an amino acid. An additional argument comes from the characterization of the bound radioactive moiety. When the phosphorylated phosvitin bands were excised, subjected to acid hydrolysis, and the nature of the labelled material thus liberated was determined by chromatography [12], the radioactive material co-migrated exclusively with phosphoserine. These findings suggest that the covalent modification of phosvitin is a result of a phosvitin kinase reaction.

3.2. *Properties of the phosvitin kinase*

As *A. axanthum* lacks an endogenous phosphorylated protein substrate, the phosvitin kinase activity in the soluble fraction of this organism could be characterized with ease. Fig.2 shows that the extent of incorporation of [γ-^{32}P] into phosvitin increased linearly for about 2 min in a reaction mixture containing NaF (10–50 mM). Incubation for longer periods of time led to a decrease in the level of phosvitin phosphorylation and the extent of decrease was dependent on the NaF concentration. When NaF was omitted from the reaction mixture, no phosphorylated phosvitin could be detected (fig.2). As NaF is known as a nonspecific phosphatase inhibitor [3], the presence of a phosphoprotein phosphatase activity in the system is suggested. When ion-exchange paper chromatography of the reaction mixture was performed as previously described [9], a vast excess (<95%) of [γ-^{32}P]ATP was found throughout the reaction period, suggesting that it is not the depletion of ATP which limits the phosvitin kinase activity. The apparent K_m for ATP to phosphorylate phosvitin catalyzed by the *A. axanthum* enzyme was 13.75 μM and V_{max} was 1.3 nmol/min per mg protein. The kinase has an optimal pH in the pH range 6.0–6.2 and requires divalent cations for activity. This requirement was best met by Mg^{2+} (50–200 μM) and to a lesser extent, by Zn^{2+}, but not by Ca^{2+}. Na^+ or K^+ added to the reaction mixture at concentrations of up to 100 mM had no significant effect on the rate and extent of phosvitin phosphorylation. NH_4^+, however, were slightly stimulatory at low concentrations and inhibitory at high concentrations (fig.3). A similar effect of NH_4^+ was previously reported for a phosvitin kinase from pig testis [13] and from ascites tumor cells [14] and a specific interaction between the enzyme and ammonium ions was suggested [13]. The phosvitin kinase was unaffected by calmodulin (6 μM) with or without 1–10 mM Ca^{2+}. The kinase was, however, markedly inhibited by heparin (0.1 μg/ml, 66% inhibition), *p*-chloromercuribenzoate (10 mM, 75% inhibition) and, to a lesser extent, by *N*-ethylmaleimide (20 mM, 20% inhibition).

In conclusion, the data presented here seem to

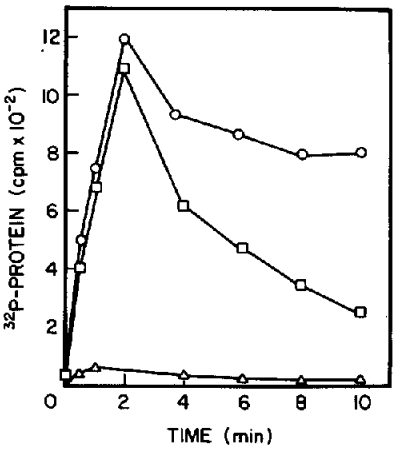

Fig.2. Kinetics of [^{32}P]phosphate incorporation into phosvitin in the presence and absence of NaF. Phosphorylation was determined following SDS-PAGE, excision of the phosvettes and counting were performed as described in section 2. (△) No NaF; (□) 10 mM NaF; (○) 50 mM NaF.

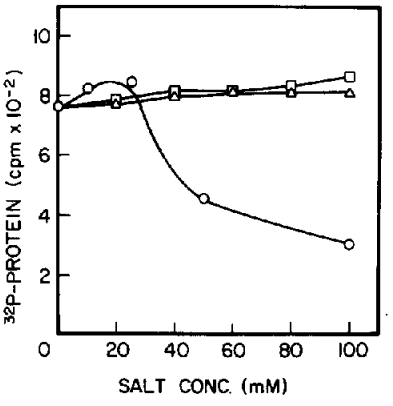

Fig.3. Effect of salt concentration on phosvitin kinase activity. Activity was assayed with increasing concentrations of salts. (△) NaCl; (□) Na_2SO_4; (○) $(NH_4)_2SO_4$.

demonstrate a protein kinase activity in *A. axanthum* that can be detected in vitro through its ability to phosphorylate phosvitin. The absence of the 55 kDa phosphorylated protein in this organism and the ability of the *A. axanthum* kinase to phosphorylate the 55 kDa protein of *M. gallisepticum* may open a way to characterize the endogenous 55 kDa phosphoprotein of the flask-shaped mycoplasma species, to study its localization within the cell and its possible physiological role in determining cell shape and gliding motility [4,14–16].

REFERENCES

[1] Cohen, P. (1985) Eur. J. Biochem. 151, 439–448.
[2] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[3] Cozzone, A.J. (1984) Trends Biochem. Sci. 9, 400–403.
[4] Platt, M.W., Rottem, S., Milner, Y., Barile, M.F., Peterkofsky, A. and Reizer, J. (1988) Eur. J. Biochem. 176, 61–67.
[5] Razin, S. and Rottem, S. (1976) in: Biochemical Analyses of Membranes (Maddy, A.M. ed.) pp.3–26, Chapman and Hall, London.
[6] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Ben-Zaguen, R., Marash, R., Loyter, A. and Milner, Y. (1977) Biochemistry 16, 3903–3909.
[9] Wallace, R.A. and Morgan, J.P. (1986) Anal. Biochem. 157, 256–261.
[10] Schmidt, M.C. and Hanna, M.M. (1986) FEBS Lett. 194, 305–308.
[11] Chen, C.C., Bruegger, B.B., Kern, C.W., Lin, Y.C., Halpern, R.M. and Smith, R.A. (1977) Biochemistry 16, 4852–4855.
[12] Manai, M. and Cozzone, A.J. (1982) Anal. Biochem. 124, 12–18.
[13] Katoh, N. and Kubo, S. (1983) Biochim. Biophys. Acta 760, 61–68.
[14] Dahmus, M.E. (1976) Biochemistry 15, 1821–1829.
[15] Townsend, R., Markham, P.G., Plaskitt, K.S. and Daniels, M.J. (1977) J. Gen. Microbiol. 100, 15–21.
[16] Neimark, H.C. (1977) Proc. Natl. Acad. Sci. USA 74, 4041–4045.