Biochimica et Biophysica Acta, 315 (1973) 347–351

BBA 66952

THE KINETIC BEHAVIOUR OF PHOSVITIN KINASE FROM RAT LIVER CYTOSOL

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(Received January 23rd, 1973)

SUMMARY

A kinetic study of a protein kinase (from rat liver cytosol), transferring P from ATP to phosvitin, is reported.

The results suggest the following:

1. The "true" substrates are the Mg–ATP^{2-} and the Mg–phosvitin complexes rather than free ATP and free phosvitin, respectively.

2. Excess of Mg^{2+} induces an inhibitory effect on the kinase reaction;

3. Such an inhibition is not observed with an only partially purified enzyme preparation.

INTRODUCTION

In a previous paper from our laboratory[1] the purification and some physicochemical properties of a proteinkinase (from rat liver cytosol) have been reported. Such an enzyme, cyclic AMP independent, is able to phosphorylate a purified cytosol phosphoprotein fraction[2] and also phosvitin, but not protamine and histones.

Using phosvitin as a phosphoprotein model, a kinetic study of the enzyme has been carried out in order to get more information about the mechanism of phosphorylation of cytosol acidic phosphoproteins, whose biological role is still an open problem.

METHODS

The crude cytosol was prepared by centrifuging for 60 min at 105 000 × *g* the 25 000 × *g* post-mitochondrial supernatant of a 0.25 M sucrose homogenate of rat liver cells[3].

The protein kinase has been purified by subjecting the cytosol to chromatography on P-cellulose, followed by Sephadex G-200 filtration at high ionic strength, as previously described[1]. The enzyme obtained at this stage (purified about eight thousand times) was stored at −20 °C and used in the kinetic experiments.

The incubation medium contained in a final volume of 1 ml: 5 μg enzyme protein, 200 μmoles Tris–HCl buffer, pH 7.5, and various amounts of substrate (phosvitin, [γ-^{32}P]ATP and Mg) as indicated in the legends to the figures. The reaction was started by addition of the enzyme sample.

After 10 min at 37 °C, the kinase reaction was stopped by adding 0.3 ml 50% trichloroacetic acid and the precipitate washed four times with 5 ml of 10% trichloroacetic acid. Finally the precipitate was transferred to a stainless planchet and counted with a thin-window Geiger counter.

The rate of kinase reaction was linear over the 10 min reaction time employed and linearity has been observed for a 40-min interval (longest time tested).

[γ-^{32}P]ATP was prepared by the method of Glynn and Chappel[4]. Phosvitin was prepared from egg yolk according the procedure of Mecham and Olcott[5].

RESULTS AND DISCUSSION

Fig. 1 shows that the Mg^{2+}-dependent kinase activity follows normal kinetics with increasing velocity as the Mg^{2+} amount is increased up to 3 μmoles.

At Mg^{2+} concentrations as high as 6–9 μmoles, an unusual saturation curve is obtained, indicating an inhibitory effect by increased Mg^{2+} at rather low ATP concentration. Such an inhibition was overcome by raising the ATP concentration to higher levels. The Mg^{2+} dependence is more evident when the data of Fig. 1 are plotted as a function of Mg^{2+} concentration at a fixed ATP level, *e.g.* 0.1 μmole (Fig. 2).

The kinase velocity in the absence of added Mg^{2+} is not zero since traces of Mg^{2+} are probably present in the enzyme preparation. This is suggested by the finding that in the presence of chelating 5 mM EDTA, the kinase activity is completely abolished.

Saturation curves similar to those of Fig. 1 are obtained by varying the concentration of phosvitin at a fixed concentration of ATP (0.1 or 0.2 μmole) (Fig. 3).

The same superimposable saturation curve is obtained with 0.2 μmole ATP and 6 μmoles Mg^{2+} or with 0.1 μmole ATP and 3 μmoles Mg^{2+}, indicating that these

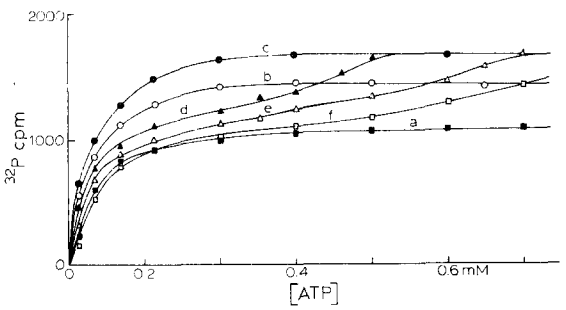

Fig. 1. Dependence of phosvitin kinase velocity on ATP concentration at various Mg^{2+} concentrations. The assay system contained in a final volume of 1 ml: 5 μg of phosvitin kinase, 200 μmoles Tris buffer, pH 7.5, 1 mg of phosvitin, various increasing concentrations of ATP and a fixed amount of Mg^{2+} as indicated: (a) ■—■, 0.6 μmoles Mg^{2+}; (b) ○—○ 1.5 μmoles of Mg^{2+}; (c) ●—●, 3 μmoles Mg^{2+}; (d) ▲—▲, 6 μmoles Mg^{2+}; (e) △—△, 9 μmoles Mg^{2+}; (f) □—□, 12 μmoles Mg^{2+}.

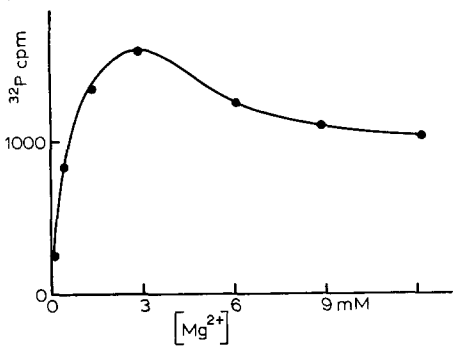

Fig. 2. Effect of Mg^{2+} concentration on phosvitin kinase reaction at a fixed level of ATP. The assay system contained in a final volume of 1 ml: 5 μg of phosvitin kinase, 200 μmoles of Tris-buffer pH 7.5, 1 mg of phosvitin, 0.1 μmole of ATP and various Mg^{2+} concentrations.

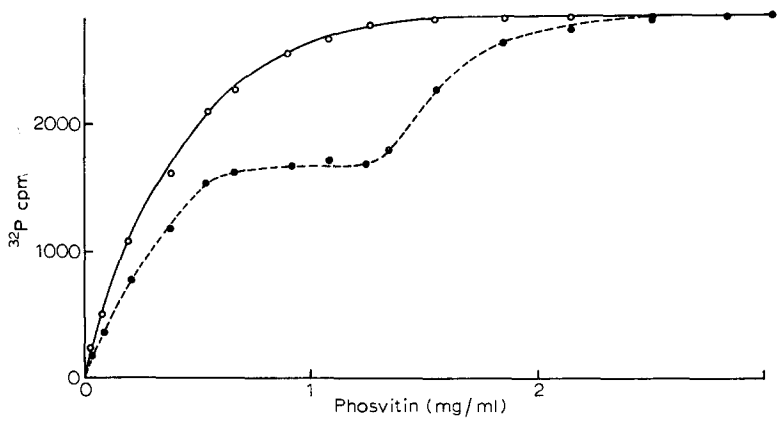

Fig. 3. Dependence of phosvitin kinase velocity on phosvitin concentration at various Mg^{2+} concentrations. The assay system contained in a final volume of 1 ml: 5 μg of phosvitin kinase, 200 μmoles of Tris buffer, pH 7.5, ATP and Mg^{2+} concentrations as indicated, various increasing amounts of phosvitin. ○—○, curve obtained either with 0.1 μmole ATP and 3 μmoles of Mg^{2+} either with 0.2 μmole ATP and 6 μmoles Mg^{2+}; ●—●, curve obtained with 0.1 μmole ATP and 6 μmoles Mg^{2+}.

latter concentrations are large enough to saturate the enzyme and to reach the maximal velocity.

When Mg^{2+} is increased up to 6 μmoles by keeping the ATP amount constant (0.1 μmole ATP) an unusual saturation curve with an intermediate plateau is obtained.

The inhibitory effect of Mg^{2+} is removed at higher phosvitin concentration*.

As a consequence (see Fig. 1 and 3), it can be concluded that under the present

* Such an unusual saturation curve is not obtained when an only partially purified enzyme preparation is used under the same conditions. It may be that in this case some other cytosol components, probably proteins bound to the enzyme[1], remove the excess of Mg^{2+}.

experimental conditions (5 μg of enzyme protein) the maximal velocity is reached when about 1 mg of phosvitin, 0.1 μmole ATP and 3 μmoles of Mg^{2+} are present in the system. Under these conditions the total concentration of phosphate groups in the system is of the same order of magnitude as that of Mg^{2+}. In this respect is must be pointed out that 1 mg of phosvitin contains about 3.0 μmoles of phosphate.

These results suggest that, in order for the reaction to occur, both phosvitin and ATP have to bind Mg^{2+}, giving rise to Mg–phosvitin and Mg–ATP complexes, which are the "true" substrates of the enzyme.

On these grounds the following facts can be explained: (1) in the presence of about 3 μmoles of Mg^{2+} maximal velocity is reached, since Mg–phosvitin and Mg–ATP complexes can be formed in a concentration sufficient to saturate the enzyme; (2) Mg^{2+} in amounts lower than 3 μmoles is the rate-limiting factor (Curves a and b of Fig. 1), because the Mg–substrate complexes concentrations are lower than those required for the saturation of the enzyme; (3) Mg^{2+} in amounts up to 6–9 μmoles displays an inhibitory effect which is removed by increasing phosvitin (Fig. 3) or ATP (Fig. 1). By increasing the substrate concentration, the excess of Mg^{2+} is bound, forming the Mg–substrate complex at concentrations higher than that required for the enzyme saturation.

That the Mg–substrate complexes are the "true" substrates is also shown by the experiments of Figs 4 and 5.

Fig. 4 shows the saturation curve obtained with phosvitin at two different levels of Mg–ATP complex (the ratio between ATP and Mg^{2+} being kept constant), whereas Fig. 5 shows the saturation curves obtained when the varied substrate is the ATP–Mg complex, at two different phosvitin levels. As can be seen from both figures, no plateau region appears, in contrast to the experiments of Figs 1 and 3. However, the saturation curve of Mg–ATP complex (Fig. 5) shows a slight sigmoidicity, and its double-reciprocal plot (inset of Fig. 5) is concave upwards. This indicates the occurrence of positive cooperative interactions between the binding sites of ATP–Mg complex. Such an interaction does not appear between the binding sites of Mg–

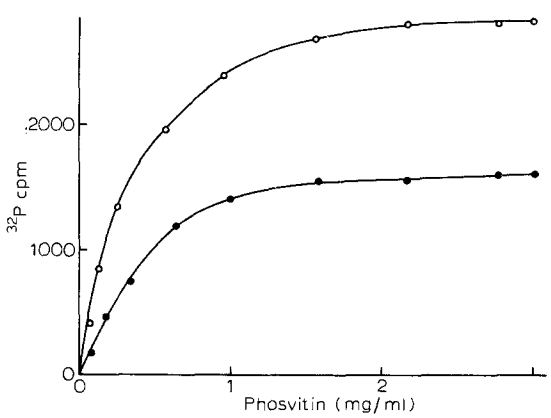

Fig. 4. Dependence of phosvitin kinase velocity on concentration of phosvitin at two different Mg–ATP complex concentrations. ●—●, 0.05 μmole ATP and 1.5 μmoles Mg^{2+}; ○—○, 0.1 μmole ATP and 3 μmoles Mg^{2+}.

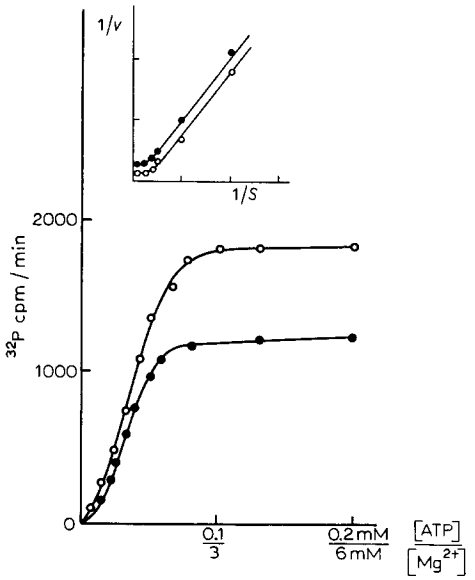

Fig. 5. Dependence of phosvitin kinase velocity on concentration of Mg–ATP complex at two different phosvitin concentrations. ●—●, 1 mg phosvitin; ○—○, 2.5 mg phosvitin. The inset shows the double-reciprocal plots.

phosvitin complex, as suggested by the absence of sigmoidicity in the saturation curve of phosvitin (Fig. 4).

The above findings described in this paper suggest that the kinase reaction occurs *via* the binding of ATP–Mg and phosvitin–Mg complex to different separate sites of the enzyme. The present results do not allow us to draw a definite conclusion about the mechanism by which the excess free Mg^{2+} inhibits the kinase reaction.

Such a mechanism is likely to be competitive in nature: the free Mg^{2+} in amounts exceeding those required to form the phosphate complex with the substrates may inhibit the binding of the Mg–substrates complex to the catalytic sites of the enzyme. However, the possibility cannot be ruled out that the free Mg^{2+} may react with the enzyme at a different site, altering the conformation of the enzyme to a less active form.

Anyway, the above results indicate that the Mg^{2+} concentration is of critical importance in regulating the activity of the cytosol protein kinase, as well as the activity of mitochondrial protein phosphatase (data to be published).

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