Biochimica et Biophysica Acta, 377 (1975) 80—83

BBA 67402

PHOSVITIN PHOSPHATE CONTENT

IMPLICATIONS FOR PROTEIN KINASE ASSAY

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(Received August 5th, 1974)

Summary

The maximal rates of the protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37) reaction studied with chicken egg yolk phosvitin as substrate are dependent on the level of dephosphorylation of phosvitin. 30% dephospho-phosvitin gives the optimal initial rates. With varying levels of dephosphorylation, the apparent K_m for the substrate also changes in a biphasic manner. If this factor is taken into account, and a suitable adjustment is made for the concentration of dephospho-phosvitin in the reaction, it is possible to achieve maximal rates for the kinase reaction with phosvitin preparations of varying levels of dephosphorylation. Such a consideration is important for comparing the results of protein kinase studies using phosvitin as the substrate.

Introduction

Egg yolk phosphoprotein, phosvitin, can serve as a model acceptor of phosphate from ATP in a variety of tissue protein phosphokinase (ATP: protein phosphotransferase, EC 2.7.1.37) reactions in vitro [1—3], and in recent years, this substrate has been used with increasing frequency in protein phosphokinase assays [4—10]. An interesting feature of this phosphoprotein is that its efficiency as a phosphate acceptor increases with dephosphorylation, so that phosvitin which is about 30% dephosphorylated gives the highest rates of phosphate incorporation. Further dephosphorylation, however, leads to a gradually decreasing phosphate acceptor activity [1—3,8]. This raises the problem of comparing the experimental results if phosvitin with the same level of dephosphorylation is not used. We have studied this point and have determined that the apparent K_m for the chicken egg yolk dephospho-phosvitin changes in a biphasic manner with the level of dephosphorylation; this offers a solution to

the problem of obtaining comparable data even if phosvitin preparations with differing levels of phosphate are used as substrate.

Experimental procedures

Phosvitin was prepared from chicken egg yolk by the method of Joubert and Cook [11], and had a phosphate content of 3.06 μmoles/mg of protein. Beef spleen phosphatase devoid of protease activity was prepared according to the procedures detailed by Revel [12] and was used to dephosphorylate phosvitin. The dephospho-phosvitin preparations were purified by column chromatography according to the procedure of Mano and Lipmann [2,3]. Protein and Pi content of the dephospho-phosvitin solutions were assayed as described earlier [2,3]. Rat ventral prostate water-soluble chromatin isolated from purified nuclei [6,9,10,13,14] possesses active protein phosphokinase(s), and was used as the source of enzyme activity to test the phosphate acceptor ability of the various dephospho-phosvitin samples. These chromatin-associated protein phosphokinase(s) are also capable of phosphorylating histones, but to a much smaller extent than phosvitin [10]. Their activity is dependent on the androgenic status of the animal, and is not increased by 3′,5′-cyclic AMP [7,9,10]. The standard reaction medium in a final volume of 1 ml consisted of 5 mM $MgCl_2$, 200 mM NaCl, 1 mM dithiothreitol, 30 mM Tris—HCl buffer, pH 7.4, at 37°C, 3 mM [γ-^{32}P]ATP as Tris salt (final specific radioactivity 3000 dpm/nmole ATP), 5—10 μg of chromatin protein, and dephospho-phosvitin as indicated. The reaction time was 30 min at 37°C. Under these conditions linear rates of incorporation of ^{32}P into dephospho-phosvitin were obtained. The reaction was terminated by the addition of 1 ml 30% (w/v) trichloroacetic acid containing 2 mM Pi and 3 mM $Na_4P_2O_7$. The precipitated protein was allowed to stand on ice for 30 min and was washed twice with 5 ml each of 15% (w/v) trichloroacetic acid containing 1 mM Pi, 1.5 mM $Na_4P_2O_7$ and 1 mM ATP, followed by four washes with 5 ml each of 15% (w/v) trichloroacetic acid containing 1 mM Pi and 1.5 mM $Na_4P_2O_7$. The radioactivity in the alkali-labile phosphates of phosvitin was measured as described earlier [5,15]. Suitable zero time controls and blanks in the absence of dephospho-phosvitin were included in the experiments.

Results and Discussion

Fig. 1 shows that as the level of dephosphorylation is increased, the efficacy of phosvitin as a phosphate acceptor in the protein kinase reaction is also enhanced. The maximal rate of incorporation of ^{32}P from [γ-^{32}P]ATP into phosvitin is observed with the level of dephosphorylation at 30%; however, as the level of dephosphorylation is increased further, the acceptor ability of phosvitin declines. This is in accord with the previous observations [1—3,8]. For the various dephospho-phosvitin preparations used in this experiment, it was possible to determined the apparent K_m value in each case. As shown in Fig. 1, the apparent K_m for phosvitin decreases markedly with increasing levels of its dephosphorylation. This change is particularly remarkable when phosvitin is dephosphorylated from 10 to 30%. The apparent K_m value is the lowest for

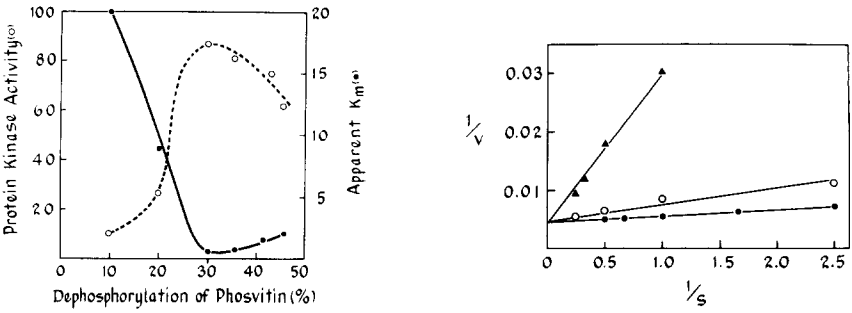

Fig. 1. Effect of the level of dephosphorylation of phosvitin on its acceptor activity and apparent K_m in the protein phosphokinase reaction. The kinase assay was performed as described under Experimental Procedures, using 2 mg of phosvitin with the indicated levels of dephosphorylation. Kinase activity (○) refers to nmoles ^{32}P incorporated into phosvitin per mg of chromatin protein per 30 min. To determine the apparent K_m values (●), the concentrations of the various dephospho-phosvitins were varied from 0.1 to 8 mg/ml in the kinase assay medium given under Experimental Procedures. The transfer of ^{32}P into dephospho-phosvitins was linear under the assay conditions. The data were plotted according to the method of Lineweaver and Burk [16] and apparent K_m values, recorded as mg of dephospho-phosvitin/ml, were determined from the graph. Several preparations of chromatin were used in these experiments; the activity of these preparations when tested in the presence of 30% dephospho-phosvitin varied by about ±8%.

Fig. 2. Double-reciprocal plot of the concentration of dephospho-phosvitin with varying levels of dephosphorylation vs kinase activity. The kinase assay was performed as described under Experimental Procedures, using phosvitin with 30% (●), 36% (○), or 20% (▲) levels of dephosphorylation. The data are plotted according to the method of Lineweaver and Burk [16]. S represents mg of dephospho-phosvitin/ml, and V represents the kinase activity as nmoles ^{32}P incorporated into phosvitin per mg of chromatin protein per h and was measured as described under Experimental Procedures. The apparent K_m values obtained from this figure were 0.36, 0.74, and 9.03 mg/ml for 30, 36, and 20% dephospho-phosvitin, respectively.

30% dephospho-phosvitin, but begins to increase again for levels of dephosphorylation above 30%. With a given preparation of dephospho-phosvitin, the increase in the rate of incorporation of phosphate with the kinase reaction follows the typical Michaelis—Menten kinetics so that saturating concentrations of the substrate can be estimated from a Lineweaver—Burk plot [16] of the data. Fig. 2 illustrates the kinetics of the protein phosphokinase reaction with phosvitin preparations at three different levels of dephosphorylation. The apparent K_m values for the three dephospho-phosvitins varied in the same manner as recorded in Fig. 1. However, it is noteworthy that the same apparent V was observed with each sample of dephospho-phosvitin. In accord with this observation, we have noted that by using a substrate concentration for various dephospho-phosvitins at about five times the respective apparent K_m value, identical rates of phosphate incorporation by the kinase are obtained. This observation may be particularly important in reporting data on protein phosphokinases derived from systems undergoing changes due to specific stimuli, e.g. testosterone-stimulated prostate [6,10] or isoproterenol-stimulated rat salivary gland [5]. Thus, we conclude that adopting this approach to the use of phosvitin as substrate can give not only optimal rates for the kinase activity under investigation, but also be of value for comparing the data when it may not be possible to always use dephospho-phosvitin with the same level of dephosphorylation.

In this study, we have examined the phosphate acceptor ability of dephospho-phosvitin with varying levels of dephosphorylation by employing rat ventral prostate chromatin as the source of kinase activity. However, is is most likely that the considerations discussed above should apply to any other source of protein phosphokinase which may be active toward dephospho-phosvitin. The fact that phosvitin at 30% levels of dephosphorylation was a more active acceptor than phosvitin at other levels of dephosphorylation for the chromatin-associated kinase, as well as the kinase activities from other sources [1—3,8], offers a strong argument in favor of such a likelihood. It should be emphasized that tissue protein phosphokinases are active toward a variety of substrates with varying degree of activity [7,17]. The considerations described above may help in further evaluating the efficacy of various acceptors in studies related to protein phosphokinase(s).

Acknowledgement

This work is supported in part by research grant No. CA-15062 from N.C.I., N.I.H., U.S.P.H.S.

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