ACTIVATION OF GLYCOGEN SYNTHETASE AND INACTIVATION OF PHOSPHORYLASE KINASE BY THE SAME PHOSPHOPROTEIN PHOSPHATASE

Franklin J. Zieve and Walter H. Glinsmann

Section on Physiological Controls, Laboratory of Biomedical Sciences, National Institute of Child Health and Human Development, Bethesda, Maryland 20014

Received December 22,1972

SUMMARY: Purified rabbit muscle glycogen synthetase D phosphatase inactivates phosphorylase kinase. The inactivation is reversed by cyclic AMP-dependent protein kinase. It is postulated that the synthetase D phosphatase is a general phosphoprotein phosphatase which dephosphorylates proteins that are phosphorylated in vivo by the cyclic AMP-dependent kinase.

A number of enzymes exist in two forms which are interconverted by phosphorylation and dephosphorylation reactions. Among these are phosphorylase (1), phosphorylase kinase (2), glycogen synthetase (3), and pyruvate dehydrogenase (4). The interconversion reactions are under hormonal control. The effects of hormones which elevate tissue cyclic AMP levels are mediated by a cyclic AMP-dependent protein kinase, which phosphorylates such diverse substrates as phosphorylase kinase (5), glycogen synthetase D (6,7), adipose tissue lipase (8,9), casein (5), and histone (10).

Relatively little attention has been paid to the regulation of the corresponding dephosphorylation reactions. Because of the very broad substrate specificity of the cyclic AMP-dependent protein kinase, we considered it likely that the reverse reactions were catalyzed by a phosphoprotein phosphatase of comparably broad specificity. The histone phosphatase described by Meisler and Langan (11), which was shown by Kato and Bishop (12) to be identical to glycogen synthetase D phosphatase, had many of the properties of the general phosphoprotein phosphatase which we had postulated. In this report we demonstrate that this phosphatase dephosphorylates and inactivates phoshorylase kinase.

MATERIALS AND METHODS

Phosphorylase kinase (40,000 units/mg) (13) and glycogen synthetase D (20 units/mg) were prepared by published methods. Glycogen synthetase D phosphatase was prepared by the method of Kato and Bishop (12) and had a specific activity of 17.6 units/mg. This corresponds to a purification of 1200-fold. Phosphorylase b (1125 Cori units/mg) was purchased from Sigma and freed of AMP by treatment with Dowex 1 resin (15). Cyclic AMP-dependent protein kinase (fraction B_2) was purified from bovine adrenal by the method of Kumon et al. (16) and separated from residual cyclic AMP-independent kinase by hydroxylapatite chromatography (16). [^{14}C]Glucose-1-P and UDP-[^{14}C] glucose were purchased from New England Nuclear.

Maximally activated phosphorylase kinase was prepared as described by Riley et al. (17). Maximally inactivated phosphorylase kinase was prepared by incubating phosphorylase kinase (300 µg) with synthetase D phosphatase (150 µg) in 100 µl of 50 mM imidazole-Cl (pH 7.4) containing 5 mM $MnCl_2$ and 1 mM dithiothreitol. After incubation for 20 min at 30°, the sample was applied to a column (0.7 x 8 cm) of Bio-gel A-1.5m equilibrated with 50 mM glycero-P (pH 6.8) containing 2 mM EDTA. The column was eluted with the same buffer. Phosphorylase kinase was eluted in the first protein peak and was free of residual phosphatase activity.

Synthetase D phosphatase assays (25 µl) contained 50 mM imidazole-Cl (pH 7.4), 5 mM $MnCl_2$, 1 mM dithiothreitol, 0.1% bovine serum albumin, 2 units/ml synthetase D, and phosphatase. After incubation for 5 min at 30°, the phosphatase reaction was stopped and the synthetase reaction was started by the addition of 25 µl of a solution containing 0.1 M Tris-Cl (pH 7.8), 0.1 M potassium phosphate (pH 7.8), 50 mM NaF, 30 mM EDTA, 1 mM dithiothreitol, 1% glycogen and 9 mM UDP-[^{14}C]glucose. After a further 10 min incubation at 30°, radioactive glycogen was isolated by the method of Thomas et al. (18).

Phosphorylase kinase phosphatase assays (50 µl) contained 50 mM imidazole-Cl (pH 7.4), 5 mM $MnCl_2$, 1 mM dithiothreitol, 0.1% bovine serum albumin, 24 µg/ml

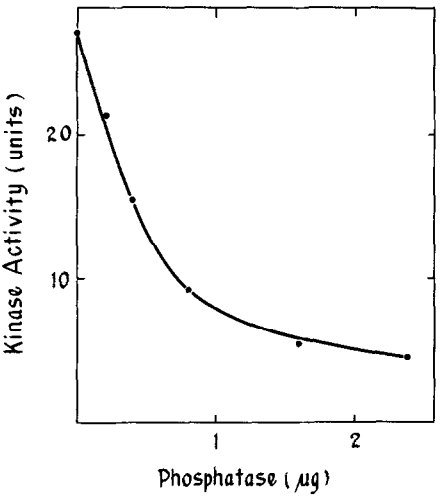

Fig. 1.

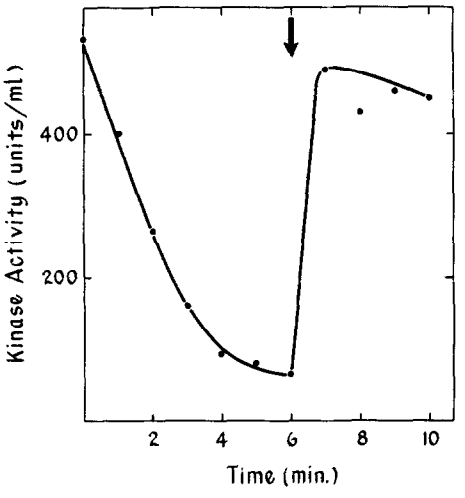

Fig. 2.

Figure 1. Inactivation of Phosphorylase Kinase by Synthetase D Phosphatase. Reaction mixtures (50 µl) contained 1.2 µg phosphorylase kinase and the amount of phosphatase shown on the abscissa.

Figure 2. Inactivation and Reactivation of Phosphorylase Kinase. The reaction mixture (200 µl) contained 4.8 µg phosphorylase kinase and 6.4 µg synthetase D phosphatase. Aliquots (5 µl) were removed at one-minute intervals and diluted for the assay of phosphorylase kinase activity. At 6 min, 10 µl of a solution (pH 7.4) containing protein kinase (6 µg) ATP (1 µmole), $MgAc_2$ (2 µmoles), cyclic AMP (50 nmoles), and potassium phosphate (0.2 µmole) were added.

maximally activated phosphorylase kinase, and phosphatase. Incubation was for 5 min at 30°, and the reactions were terminated by the addition of 500 µl of cold 15 mM cysteine (pH 7.0) containing 1 mM potassium phosphate. Phosphorylase kinase assays were carried out at pH 6.8 as described by Krebs (19) with the following modifications: (a) The volume of the kinase reaction mixture was reduced to 25 µl; (b) [^{14}C]glucose-1-P was used in the phosphorylase assay; (c) the volume of the phosphorylase reaction mixture was 60 µl; (d) radioactive glycogen was isolated by the method of Thomas et al. (18).

RESULTS AND DISCUSSION

When activated phosphorylase kinase was incubated with purified synthetase D phosphatase, a rapid decrease in kinase activity occurred. Fig. 1 shows the decrease in kinase activity in the presence of varying amounts of synthetase D

phosphatase. With the largest quantity of phosphatase used (2.4 μg), kinase activity was reduced by 84%. Kinase activity at pH 8.2, which measures both the active and the inactive forms of the enzyme (2), was not reduced by synthetase D phosphatase (data not shown).

It was important to determine whether the inactivation of phosphorylase kinase by synthetase D phosphatase involved dephosphorylation of the kinase to its inactive form or whether it involved a different mechanism of inactivation (e.g. proteolysis). If dephosphorylation were involved, it should be possible to reverse the inactivation with cyclic AMP-dependent protein kinase (5). The experiment shown in Fig. 2 was designed to test the reversibility of the inactivation. Activated phosphorylase kinase was incubated with synthetase D phosphatase for 6 min at 30°, at which point kinase activity had fallen to less than 13% of its initial level. ATP, Mg^{2+}, cyclic AMP, and protein kinase were then added, and incubation was continued. Within 1 min after the addition of protein kinase, phosphorylase kinase activity had risen to more than 90% of its initial level. This rapid reactivation by the protein kinase strongly suggests that synthetase D phosphatase inactivates phosphorylase kinase by dephosphorylation.

It was possible, of course, that the dephosphorylation of phosphorylase kinase was not catalyzed by synthetase D phosphatase itself, but by a separate phosphatase which was present in the synthetase D phosphatase preparation. If phosphorylase kinase phosphatase and synthetase D phosphatase were different enzymes, one would not expect the ratio of the two activities to remain constant during the purification procedure. Table 1 lists the phosphatase activities of the fractions obtained during the purification of synthetase D phosphatase. After a purification of more than 1000-fold, the ratio of phosphorylase kinase phosphatase to synthetase D phosphatase was essentially unchanged. This finding suggests that the two activities are due to the same enzyme.

More direct evidence in support of this conclusion was obtained from studies of the effect of activated phosphorylase kinase on the conversion of

Table 1

Phosphatase Activities of Synthetase D Phosphatase Fractions

Fraction	Phosphatase Activity (units/mg)	
	Phosphorylase Kinase[a]	Synthetase D[b]
78,000 g supernatant	0.04	0.014
DEAE-cellulose	9.6	2.1
Sephadex G-200	56	17.6

[a]A unit of phosphorylase kinase phosphatase is the amount which inactivates 100 units of phosphorylase kinase per minute at 30°.

[b]A unit of synthetase D phosphatase is the amount which converts one unit of synthetase D to synthetase I per minute at 30°.

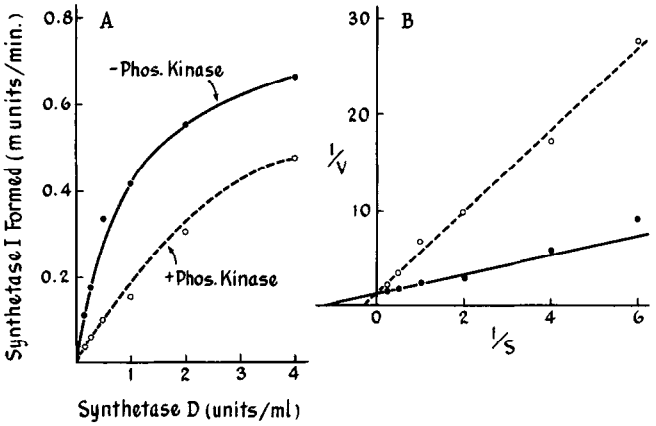

Figure 3. Effect of Activated Phosphorylase Kinase on the Conversion of Synthetase D to Synthetase I. Reaction mixtures (25 µl) contained 0.03 µg synthetase D phosphatase and 0.04% glycogen in addition to the components listed under MATERIALS AND METHODS. Where indicated, 1.2 µg activated phosphorylase kinase was added. Incubation was for 3 min at 30°.

synthetase D to synthetase I. As demonstrated in Fig. 3, phosphorylase kinase inhibited the synthetase D phosphatase reaction. Furthermore, the inhibition was clearly competitive in nature (Fig. 3B). It therefore appears that phosphorylase kinase and synthetase D are substrates for the same phosphatase.

The marked inhibition of synthetase D phosphatase activity was only

Table 2

Effect of Active and Inactive Phosphorylase Kinase on Synthetase D Phosphatase Activity

Additions	Synthetase I Formed (munits)
None	5.9
Active phosphorylase kinase	2.5
Inactive phosphorylase kinase	5.7

Reaction mixtures (25 µl) contained 50 munits synthetase D, 0.06 µg synthetase D phosphatase, and 2 µg phosphorylase kinase. Maximally activated and maximally inactivated phosphorylase kinase were prepared as described under MATERIALS AND METHODS.

produced by the active form of phosphorylase kinase (Table 2). Inactive phosphorylase kinase at the concentration tested had no effect on the formation of synthetase I. The same amount of active phosphorylase kinase inhibited synthetase I formation by more than 50%.

We conclude that the inactivation of phosphorylase kinase and the activation of glycogen synthetase are catalyzed by the identical phosphoprotein phosphatase. This same phosphatase also dephosphorylates histone (12). All three of these phosphoproteins are formed by the action of cyclic AMP-dependent protein kinase (5-7,10). It appears likely to us that synthetase D phosphatase is a rather general phosphoprotein phosphatase which dephosphorylates proteins that are phosphorylated in vivo by the cyclic AMP-dependent kinase.

ACKNOWLEDGMENTS

We are grateful to Dr. M. Mitsunaga for the preparation of cyclic AMP-dependent protein kinase and to Dr. J.S. Bishop for communicating his purification procedure to us prior to its publication.

REFERENCES

1. Krebs, E.G., and Fischer, E.H., Biochim. Biophys. Acta 20, 150 (1956).
2. Krebs, E.G., and Fischer, E.H., Ann. N.Y. Acad. Sci. 88, 378, (1960).
3. Rosell-Perez, M., Villar-Palasi, C., and Larner, J., Biochemistry 1, 763 (1962).

4. Wieland, O., and Siess, E., Proc. Nat. Acad. Sci. 65, 947 (1970).
5. Walsh, D.A., Perkins, J.P., and Krebs, E.G., J. Biol. Chem. 243 3763 (1968).
6. Schlender, K.K., Wei, S.H., and Villar-Palasi, C., Biochim. Biophys. Acta 191, 272 (1969).
7. Soderling, T.R., Hickenbottom, J.P., Reimann, E.M., Hunkeler, F.L., Walsh, D.A., and Krebs, E.G., J. Biol. Chem. 245, 6317 (1970).
8. Corbin, J.D., Reimann, E.M., Walsh, D.A., and Krebs, E.G., J. Biol. Chem. 245, 4849 (1970).
9. Huttunen, J.K., Steinberg, D., and Mayer, S.E., Biochem. Biophys. Res. Commun. 41, 1350 (1970).
10. Langan, T.A., Science 162, 579 (1968).
11. Meisler, M.H., and Langan, T.A., J. Biol. Chem. 244, 4961 (1972).
12. Kato, K., and Bishop, J.S. J. Biol. Chem. 247, 7420 (1972).
13. DeLange, R.J., Kemp, R.G., Riley, W.D., Cooper, R.A., and Krebs, E.G., J. Biol. Chem. 243, 2200 (1968).
14. Brown, N.E., and Larner, J., Biochim. Biophys. Acta 242, 69 (1971).
15. Gilboe, D.P., and Nuttall, F.Q., Anal. Biochem. 47, 28 (1972).
16. Kumon, A., Nishiyama, K., Yamamura, H., and Nishizuka, Y., J. Biol. Chem. 247, 3726 (1972).
17. Riley, W.D., DeLange, R.J., Bratvold, G.E., and Krebs, E.G., J. Biol. Chem. 243, 2209 (1968).
18. Thomas, J.A., Schlender, K.K., and Larner, J., Anal. Biochem. 25, 486 (1968).
19. Krebs, E.G., in E.F. Neufeld and V. Ginsburg (Editors), Methods in Enzymology, Vol. VIII, Academic Press, New York (1966), p. 543.