STUDIES ON THE MECHANISM OF ACTIVATION AND DEACTIVATION OF PHOSPHORYLASE KINASE FROM RABBIT SKELETAL MUSCLE

BY

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A Thesis

Presented to the

Faculty of Graduate Studies

The University of Manitoba

In Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

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ACKNOWLEDGEMENT

I would like to express my gratitude to Dr. Jerry H. Wang for his close supervision and enthusiasm during the course of this work. His comments on this manuscript during its preparation are highly appreciated.

I am grateful to Drs. K. Dakshinamurti and R. L. Khandelwal for reading this manuscript before it was typed in its final form and for making valuable suggestions. I am also thankful to Dr. Khandelwal for helpful discussions concerning the dephosphorylation of activated phosphorylase kinase.

Finally, Miss B. Persaud is to be thanked for typing this manuscript.

ABSTRACT

In this study the activation of phosphorylase kinase by the protein kinase-catalyzed reaction and deactivation of different phosphorylated forms of the enzyme by a homogeneous preparation of a phosphoprotein phosphatase were investigated. The stimulation of the nonactivated kinase by organic solvents and fatty acids was also examined.

In the presence of 10 mM Mg^{2+}, phosphorylase kinase can be activated to very high levels by the cAMP-dependent protein kinase-catalyzed reaction. These levels are much higher than those observed previously when the reaction was studied at 1 to 2 mM Mg^{2+} (Cohen, P. (1973) Eur. J. Biochem. 34, 1-14; Hayakawa, T. Perkins, J. P., and Krebs, E. G. (1973) Biochemistry 12, 574-580). The activation of phosphorylase kinase seems to occur in two phases. At low Mg^{2+} (1 to 2 mM) only the first phase is manifested and involves the incorporation of 2 moles of phosphate, 1 mole into each of subunits A and B. This results in a 30 to 50 fold activation of the enzyme. At high Mg^{2+} (10 mM), 7 to 9 moles phosphate could be incorporated, the additional sites phosphorylated being on subunit A. The extent of activation achieved by the kinase is 2 to 3 times higher than that observed when the reaction is studied at low Mg^{2+}. With substrates of protein kinase other than phosphorylase kinase the same extent of phosphorylation is achieved at low and high Mg^{2+}. This suggests that Mg^{2+} may be altering the conformation of phosphorylase kinase in such a way as to render more phosphorylation sites accessible to protein kinase.

Phosphorylase kinase activation could be reversed by a phospho-

protein phosphatase of broad substrate specificity (Khandelwal, R. L., Vandenheede, J. R., and Krebs, E. G. (1976) J. Biol. Chem. 251, 4850-4858). This phosphatase incompletely dephosphorylates phosphorylase kinase. A clearly defined resistant phase is observed regardless of the initial phosphorylation state of phosphorylase kinase used as a substrate for the phosphatase. Mg^{2+} or Mn^{2+} ions do not stimulate the dephosphorylation of this resistant phase. Examination of the subunit dephosphorylation pattern revealed that the release of ^{32}P radioactivity from subunit B is very rapid compared to subunit A. The resistant phase seems to reflect the dephosphorylation mainly of subunit A. For phosphorylase kinase containing 0.5 to 2 moles of phosphate, subunit B dephosphorylation results in the almost complete reversal of the kinase to the nonactivated state. However, phosphorylase kinase containing 5 to 10 moles of phosphate lost only 50 to 60% of its activity when subunit B dephosphorylation was complete. The remaining 40 to 50% activity is lost when sites on subunit A are dephosphorylated. These sites on subunit A can be rapidly dephosphorylated by a phosphatase present in both liver and skeletal muscle homogenates.

The activity of nonactivated phosphorylase kinase can be stimulated by high concentrations (1 M) of various organic solvents at pH 6.8 In increasing order of effectiveness methanol, 1-propanol, methyl isobutyl ketone, ethyl formate, formamide, ethanol, dimethyl sulfoxide, 2-propanol, tetrahydrofuran and acetone stimulated the kinase activity from 3-fold (methanol) to 28-fold (acetone). At higher concentrations ethanol was the most effective solvent. At 1.72 M a 46-fold enhancement in kinase activity was achieved. If phosphorylase kinase is

first activated by phosphorylation only a small degree of stimulation of its activity by organic solvents is observed. The stimulation of the nonactivated kinase by organic solvents is largely, but not totally, reversible upon removal of the solvent. In the presence of organic solvents, phosphorylase kinase is not dissociated to lower M. W. species nor does it lose its dependency on Ca^{2+} ions for activity. The stimulation of casein phosphorylation and the autophosphorylation of the kinase by organic solvents suggests that the stimulatory effect of organic solvents is directly on the phosphorylase kinase molecule. Further, the unsaturated fatty acids, palmitoleic and oleic acids, can also enhance the activity of nonactivated phosphorylase kinase. A 6-fold stimulation could be achieved at low concentrations (0.8 mM) of these fatty acids. These studies suggest that phosphorylase kinase may exhibit high activity without undergoing covalent modification.

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ABBREVIATIONS

5' - AMP, adenosine 5' - monophosphate

ATP, adenosine triphosphate

cAMP, adenosine 3', 5' - monophosphate

cGMP, guanosine 3', 5' - monophosphate

DTNB, 5, 5' - dithiobis -(2-nitrobenzoic acid)

EDTA, ethylenediamineteraacetic acid

EGTA, ethylene glycol bis (β- aminoethyl ether) - N, N' - tetraacetic acid

Mes, 2-(N-morpholino) - ethanesulfonic acid

Monomeric Unit, the minimal M. W. of phosphorylase kinase, 318,000 (116)

M. W., molecular weight

P_i, inorganic phosphate

SDS, sodium dodecyl sulfate

Tes, N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid

TN-I, inhibitory subunit of troponin

TN-T, tropomyosin-binding subunit of troponin

I. INTRODUCTION

Phosphorylase kinase (ATP: phosphorylase phosphotransferace, EC 2.7.1.38) is of pivotal importance in the enzyme system responsible for the glycogenolysis in skeletal muscle (1, 2, 3,). The enzyme is composed of three types of subunits, A, B, C and has the molecular structure A_4 B_4 C_4 (4). It can exist in nonactivated and activated states. The nonactivated enzyme can be converted to the activated form by protein phosphorylation. Two mechanisms have been elucidated for the enzyme activation. One of these is catalyzed by the cAMP-dependent protein kinase and the other by phosphorylase kinase itself (5). These mechanisms have been studied in great detail (4, 6, 7, 8). Both modes of activation involve the phosphorylation of subunits A and B. The incorporation of 1 mole of phosphate into each of subunits A and B by the protein kinase-catalyzed reaction result in a 30 to 50-fold activation of phosphorylase kinase. Enzyme activation seems to correlate best with the phosphorylation of subunit B (4). The autoactivation of phosphorylase kinase results from the incorporation of 7 to 9 moles of phosphate per monomeric unit of the kinase with subunit A phosphorylation being greater than that of subunit B in the ratio 2.5:1 (7). Enzyme activation can reach as high as 200-fold (8) and seems to depend on the phosphorylation of both subunits A and B (7).

An evaluation of the activation of phosphorylase kinase resulting from the autocatalytic and protein kinase-catalyzed reactions revealed that non-identical sites may be phosphorylated on the kinase by the two different mechanisms (7). While trying to reproduce this and similar results it was observed that in the presence of high concentrations of Mg^{2+}, phosphorylase kinase can be activated by the protein kinase-catalyzed reaction to higher levels than previously observed (9). In

fact, total phosphate incorporation, the fold enzyme activation and the phosphorylation pattern of the subunits are very similar to that observed for activation by the autocatalytic mechanism. The activation of phosphorylase kinase catalyzed by protein kinase at high Mg^{2+} concentrations has been characterized and compared to the autoactivation of the enzyme. The possible mechanism of the Mg^{2+} effect is discussed.

Phosphorylase kinase activation resulting from phosphorylation of the enzyme can be reversed by phosphoprotein phosphatase(s). Preliminary studies (10, 11, 12) on the reversal of the kinase activation by protein dephosphorylation have been attempted. However, detailed analysis of the dephosphorylation of phosphorylase kinase phosphorylated to different levels and by different mechanisms have not been done. In the present study phosphorylase kinase labelled at approximately 1, 2 or 5 to 7 sites by the cAMP-dependent protein kinase and 9 to 10 sites by the autocatalytic reaction were used as substrates for a homogeneous preparation of phosphoprotein phosphatase (13). The loss of enzyme activity was correlated with the dephosphorylation of particular subunits. The influence of the initial phosphorylation state of the phosphorylase kinase molecule on enzyme deactivation will be considered. An evaluation will be made on the importance of the phosphorylation state of individual subunits in influencing the dephosphorylation of other subunits. Also the preference of the phosphatase for kinase phosphorylated by the protein kinase-catalyzed reaction or by autophosphorylation will be assessed.

The possibility of an efficient activation of phosphorylase kinase by a non-covalent mechanism was also investigated. Previously glycogen, heparin (14), Mg^{2+} (15, 16) and neutral salts (17) were shown to

stimulate nonactivated phosphorylase kinase by various small extents. In addition, Ca^{2+} ions have been shown to be essential for the kinase activity (5, 18, 19). Hence, it has been hypothesized that glycogenolysis can be coupled to muscular contraction by the stimulation of phosphorylase kinase by Ca^{2+} ions released from the sarcoplasmic reticulum. However, it has been demonstrated that phosphorylase kinase is not converted to the activated form during the electrical stimulation of muscle (20, 21, 22) and the nonactivated kinase shows relatively low activity even in the presence of saturating amounts of Ca^{2+}. Such low activity cannot account for the rapid conversion of phosphorylase b to the a form observed during neural stimulation of muscle. Hence the possibility was considered that activators of phosphorylase kinase may exist that allow the enzyme to exhibit high activity under certain conditions without the need to be activated by phosphorylation. During the course of this study it was observed that high concentrations of various organic solvents can stimulate phosphorylase kinase in a largely reversible manner. The fold stimulation of the kinase activity achieved is similar to that obtained when two sites on the enzyme are phosphorylated (4). In addition unsaturated fatty acids were found to stimulate the nonactivated kinase 6 to 7 fold at low concentrations. The significance of these findings is discussed.

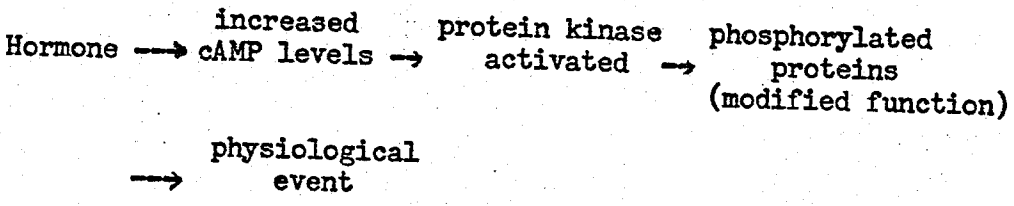
II. LITERATURE REVIEW

The literature review will be confined largely to an examination of the properties and detailed regulation of phosphorylase kinase. Hence, Section II. A is meant to be only a general introduction to the already large and rapidly expanding field of protein phosphorylation. More detailed accounts on the properties and regulation of protein kinases have been given elsewhere (3, 23, 24, 25, 26). Studies on some of the cellular processes controlled by phosphorylation-dephosphorylation reactions have also been documented. These include glycogen metabolism (2, 26, 27, 28,), lipid metabolism (29-34), protein biosynthesis (35-39), nervous function (40-44), membrane transport (45-49, 50-53) and smooth muscle contraction (54-56), among others.

A. Protein Phosphorylation and the Regulation of Cellular Function

Studies on glycogen phosphorylase revealed for the first time that protein phosphorylation is a potentially important mechanism for controlling cellular function. In the early stages of their studies on muscle phosphorylase, Cori and coworkers recognized that the enzyme exists in two enzymatically interconvertible forms called phosphorylase b and phosphorylase a, (57 - 58). Phosphorylase a is the physiologically active form of the enzyme (59). The conversion of phosphorylase b to the a form was shown to be catalyzed by a protein kinase (later called phosphorylase b kinase) and to involve phosphorylation of the enzyme (60) . The enzyme catalyzing the reverse reaction was called phosphorylase phosphatase (61).

Since the original discovery of cAMP by Sutherland and Rall (62) this nucleotide has been shown to affect numerous cellular processes (3, 63, 64). Many hormones can increase the levels of cAMP in target cells (64) and the nucleotide has been proposed to act as a "second messenger" (65) in these cells. The mechanism by which cAMP carries out its role as second messenger was solved by the discovery of a protein kinase (66) that could be activated by the nucleotide. The broad substrate specificity of the cAMP-dependent protein kinase (66) as well as its ubiquitous distribution (67) has led to the hypothesis that all actions of cAMP in higher organisms are mediated by protein kinase-catalyzed phosphorylation reactions (3, 67) . Scheme A outlines a postulated mechanism (3) to explain the mode of action of those hormones that cause an increase in the cellular level of cAMP.



Scheme A

Protein kinases have arbitrarily been divided into two classes on the basis of their response to cyclic nucleotides. The cyclic nucleotide-dependent protein kinases are active only in the presence of these nucleotides whereas the independent kinases are equally active either in the presence or absence of cyclic nucleotides. With the exception of a few cases, little is known about the regulation of the cyclic nucleotide independent kinases even though these have been purified and characterized

in many cases (68-76, 77) . The regulation of phosphorylase kinase is now understood in detail (see below). A cyclic nucleotide independent kinase that has been termed glycogen synthase kinase 2(71, 78, 79) was purified from skeletal muscle and shown to catalyze the phosphorylation and inactivation of glycogen synthase (79-81). A protein kinase that is converted from an inactive to an active form by phosphorylation and is involved in the control of protein synthesis in rabbit reticulocytes has been chacterized (37, 38, 75, 76, 82).

The cyclic nucleotide-dependent protein kinases can be subdivided on the basis of whether they are cAMP-dependent or cGMP-dependent. These kinases have a high affinity for cAMP and cGMP, respectively. Both classes of protein kinases show a wide distribution among mammalian tissues (67,83,84). In addition, these kinase activities have been detected in both the soluble and particulate fractions of cells (85-90). The soluble enzymes from a number of tissues have been extensively purified and characterized.

The cAMP-dependent enzyme can be subdivided into type I and type II protein kinases (91). The type I kinase from skeletal muscle (26, 92, 93) and the type II kinase from heart (23,94,95) have been purified to homogeneity. The activation of these protein kinases by cAMP was shown to involve the binding of the nucleotide to the holoenzyme and the subsequent dissociation of the latter into catalytic (C) and regulatory (R) subunits (96-98) as shown in Scheme B.

$$R_2 C_2 + 2\ cAMP \longrightarrow R_2 \cdot (cAMP)_2 + 2C$$

Scheme B