Activation of Glycogen Phosphorylase Kinase by a Calcium-activated, Cyclic Nucleotide-independent Protein Kinase System*

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SUMMARY

A protein kinase, which was produced from its proenzyme occurring in rat brain upon limited proteolysis by a Ca²⁺-dependent protease from the same tissue (Inoue, M., Kishimoto, A., Takai, Y., and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7016-7018, was capable of phosphorylating α and β subunits of rabbit skeletal muscle glycogen phosphorylase kinase, resulting in a marked enhancement of the enzymatic activity. This protein kinase was entirely independent of cyclic nucleotides and differed from the catalytic subunit of cyclic AMP-dependent protein kinase. The activation of phosphorylase kinase by this active protein kinase was not inhibited by a protein inhibitor of cyclic AMP-dependent protein kinase, nor by ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid, which prevented autophosphorylation of phosphorylase kinase. The proenzyme was distinguishable from cyclic nucleotide-dependent protein kinases, since it did not bind cyclic AMP and cyclic GMP, and was inactive in the phosphorylation and activation of phosphorylase kinase both in the presence and absence of these cyclic nucleotides. Neither the protein kinase nor its proenzyme showed phosphorylase kinase activity. Available evidence indicates that the Ca²⁺-activated, cyclic nucleotide-independent protein kinase system is able to phosphorylate muscle glycogen phosphorylase kinase, resulting in a marked enhancement of the enzymatic activity; the proenzyme was unable to activate phosphorylase kinase both in the presence and absence of cyclic nucleotides. The protein kinase which is produced from the proenzyme will be tentatively referred to in this paper as protein kinase A, and cyclic AMP- and cyclic GMP-dependent protein kinases as protein kinase A and protein kinase G, respectively.

Protein kinase M was routinely assayed by measuring the incorporation of radioactive γ-phosphate of ATP into calf thymus histone (see the legend to Fig. 1), and was purified partially from the soluble fraction of bovine cerebellum as described (9). The specific activity of the enzyme was about 2,500 units/mg of protein. The catalytic subunit of protein kinase A with a specific activity of about 25,000 units/mg of protein was prepared from bovine cerebellum by the method described elsewhere (9). One unit of these kinases was defined as that amount of enzyme which incorporated 1 pmol of phosphate from ATP into calf thymus histone per min under the respective assay conditions (9, 12). The proenzyme of protein kinase M and the Ca²⁺-dependent protease employed for the present studies were purified from rat brain as described elsewhere (11). The proenzyme and protease employed were free of each other and of other interfering enzymes and endogenous phosphate acceptor proteins. Glycogen phosphorylase b and phosphorylase kinase were purified from rabbit skeletal muscle by the method of Fischer and Krebs (14) and Cohen (15), respectively. Protein inhibitor of protein kinase A was prepared from rabbit skeletal muscle by the method of Walsh et al. (16). Calf thymus histone was prepared by the method of Johns (17). [γ-³²P]ATP was pre-

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pared by the method of Glynn and Chappell (18). Other chemicals were obtained from commercial sources.  

Activation of phosphorylase kinase was assayed at 30° in principle as described by Cohen (15). The reaction mixture (0.1 ml) initially contained 2.5 μmol of Tris/HCl at pH 7.5, 0.5 μmol of magnesium acetate, 0.1 μmol of ATP, 3 μmol of 2-mercaptoethanol, 0.1 μmol of EGTA, 45 μg of phosphorylase kinase, and either protein kinase M or the catalytic subunit of protein kinase A. After incubation for 5 min (1st incubation), a 10-μl aliquot of the mixture was diluted with 1.0 ml of ice cold 50 mM glycerol 1-phosphate at pH 6.8 containing 2 mM EDTA and 10 mM 2-mercaptoethanol. Then, a 10-μl aliquot of the resulting solution was added as immediately as possible to the assay mixture (0.19 ml) for phosphorylase kinase, which contained 6 μmol of glycerol 1-phosphate at pH 6.8, 2 μmol of magnesium acetate, 0.4 μmol of ATP, 0.04 μmol of calcium chloride, 1.5 μmol of 2-mercaptoethanol, and 30 μg of crystalline phosphorylase b (free of 5'-AMP). After incubation for 5 min at 30° (2nd incubation), a 20-μl aliquot of the incubation mixture was directly added to the mixture (0.38 ml) for phosphorylase assay, which contained 10 μmol of glycerol 1-phosphate at pH 6.8, 2 μmol of ATP, (3 to 4 x 109 cpm), 0.8 μmol of EDTA, and 2 mg of glycogen. The mixture was incubated for an additional 10 min at 30° (3rd incubation). The phosphorolytic reaction of glycogen was finally stopped, and P, was concomitantly precipitated with 2.5 μmol of P, as carrier to a triethylamine-phosphomolybdate complex by the addition of 1 ml of a mixture (10% perchloric acid:10% ammonium molybdate:0.2 M triethylamine, 4:2:1) by the method of Sugino and Miyoshi (19). Radioactive glucose 1-phosphate remaining in the supernatant fluid was determined by the method described previously (20). All reactions mentioned above proceeded linearly with time during the three incubations leading to the measurement of phosphorylase activity.

Autocatalytic activation of phosphorylase kinase was assayed at 30° in a reaction mixture (0.1 ml) which contained 2.5 μmol of Tris/HCl at pH 8.3, 1 μmol of magnesium acetate, 0.3 μmol of ATP, 0.01 μmol of calcium chloride, 3 μmol of 2-mercaptoethanol, 0.2 μg of protein inhibitor, and 45 μg of phosphorylase kinase. After incubation for 3 min, a 10-μl aliquot of the incubation mixture was diluted with 1.0 ml of ice cold 50 mM glycerol 1-phosphate at pH 6.8, containing 2 mM EDTA and 10 mM 2-mercaptoethanol. The subsequent assay for phosphorylase kinase was as described above. Protein kinase M was unknown by the method of Lowry et al. (21) with bovine serum albumin as a standard.

Table I shows effects of the proenzyme and protein kinases M and A on phosphorylase kinase in the presence and absence of ATP. Protein kinase M showed an ability to activate phosphorylase kinase in the presence of ATP, whereas the proenzyme was practically inactive in this capacity. The relative efficiencies of protein kinases M and A were almost identical. In the absence of ATP, both protein kinases M and A were inactive, suggesting that the activation by protein kinase M was due to the phosphorylation of the enzyme molecule as described for the activation by protein kinase A. This activation reaction by protein kinase M was not stimulated nor inhibited by cyclic AMP or cyclic GMP. Under these conditions, the proenzyme and protein kinase M did not show by themselves phosphorolysis and phosphorylase kinase activities.

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2 The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)N,N′-tetraacetic acid; SDS, sodium dodecyl sulfate.

3 See Table I footnotes.

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Table I Effects of proenzyme, protein kinases M and A on activation of phosphorylase kinase

<table>
<thead>
<tr>
<th>Protein kinase</th>
<th>Phosphorylase kinase</th>
<th>Glucose 1-phosphate</th>
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<tbody>
<tr>
<td></td>
<td>Without ATP*</td>
<td>With ATP*</td>
</tr>
<tr>
<td>None</td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>1,990*</td>
</tr>
</tbody>
</table>

* Indicate the presence and absence of ATP during the 1st incubation.

† This small activity was presumably due to autophosphorylation (autoactivation) of phosphorylase kinase or to the intrinsic activity of phosphorylase kinase at pH 6.8 (15) during the 2nd incubation.

All 2nd incubation mixtures contained Ca²⁺ and ATP (see text).

To examine further whether phosphorylase kinase was actually phosphorylated and activated by protein kinase M, experiments were performed starting with the proenzyme and protease. When the proenzyme was preincubated with the Ca²⁺-dependent protease under the conditions specified in Fig. 1, the enzymatic activities to phosphorylate histone and phosphorylase kinase were increased together, as assayed by measuring acid-precipitable radioactivity. Concomitantly, the enzymatic activity to activate phosphorylase kinase was increased as assayed by three sequential incubations leading to glycogenolysis as described above. All these enzymatic activities did not appear if Ca²⁺ was omitted from the preincubation mixture. Both proenzyme and protease were absolutely necessary during the preincubation (data not shown); therefore, it is not possible that the Ca²⁺-dependent protease directly phosphorylase kinase through proteolysis. In another set of experiments, aliquots of the preincubation mixture were taken at various periods of time and then employed for phosphorylation of phosphorylase kinase with [γ-³²P]ATP under the conditions given in Fig. 2. The resulting radioactive phosphorylase kinase was subjected to polyacrylamide gel electrophoresis in the presence of SDS, and densitometric tracing and determination of radioactivity incorporated into each subunit were performed. As illustrated in this figure, both α and β subunits of phosphorylase kinase were labeled with time; the rate of labeling of the β subunit appeared to be faster than that of the α subunit. Under these conditions, roughly 1 mol of phosphate was incorporated into every mol.
of both α and β subunits. It has been recently reported that at 10 mM Mg\(^{2+}\), protein kinase A incorporated several moles of phosphate into each monomeric unit (22). Exact stoichiometry as well as the sites of phosphorylation by protein kinase M will be explored by further investigations.

The activation of phosphorylase kinase by protein kinase M was not susceptible to the protein inhibitor of protein kinase A, whereas that by protein kinase A was completely depressed by the inhibitor under the same conditions as shown in Fig. 3. EGTA did not block the activation of phosphorylase kinase; this ruled out the possibility that the observed activation simply represented autocatalytic activation of phosphorylase kinase. The optimum concentration of Mg\(^{2+}\) for the activation of phosphorylase kinase by protein kinases M and A was 5 to 10 mM, whereas that for autocatalytic activation was 35 to 50 mM. The \(K_m\) value for ATP was about 5 \times 10^{-6} M for both protein kinases M and A, and 4 \times 10^{-3} M for the autoproteolytic reaction as judged by activation of phosphorylase kinase.

It has been described that both protein kinase A (23) and protein kinase G (10) may be activated by limited proteolysis with trypsin and that protein kinase G shows a catalytic activity apparently similar to that of protein kinase A (24-26). Nevertheless, before being treated with proteases, the proenzyme was unable to activate phosphorylase kinase, nor able to phosphorylate histone over a wide range of concentrations of cyclic AMP or cyclic GMP (10^{-7} to 10^{-4} M). In

![Fig. 2. SDS-polyacrylamide gel electrophoresis of phosphorylase kinase phosphorylated by protein kinase M. The proenzyme (32 µg) was preincubated with Ca\(^{2+}\)-dependent protease (10 µg) at 20°C in 0.2 ml of 10 mM Tris/Cl at pH 7.5, containing 2 mM CaCl\(_2\) and 5 mM 2-mercaptoethanol. At various times indicated, a 40-µl aliquot was directly added to another reaction mixture (0.16 ml) containing 5 µmol of Tris/Cl at pH 7.5, 20 nmol of [γ-\(^{32}\)P]ATP (2.5 \times 10^5 cpm/nmol), 1 µmol of magnesium acetate, 6 µmol of 2-mercaptoethanol, 0.2 µmol of EGTA, 0.4 µg of protein inhibitor, and 369 µg of phosphorylase kinase. After incubation for 40 min at 30°C, the reaction was stopped by the addition of 50 µl of ice cold 0.25 M EDTA at pH 7.0. The radioactive phosphorylase kinase was isolated and analyzed with 5% polyacrylamide gel electrophoresis in the presence of SDS by the method described by Cohen (15). Protein (0.2 µg) was applied to each gel, and densitometric tracing was performed at 660 nm using an ISCO gel scanner, model 659, after staining with Coomassie brilliant blue. Radioactivity in each protein band was determined by transverse sectioning of the gel into 1.5-mm widths followed by counting individual slices directly in 10 ml of distilled water with a Packard Tri-Carb liquid scintillation spectrometer, model 3320. A, B, and C, radioactivity at 0, 2, and 8 min preincubation, respectively. During the subsequent 40-min incubation, 0.6, 1.3, and 1.7 mol of radioactive P, were incorporated, respectively, into every monomeric unit of phosphorylase kinase. The small peaks in A represent the radioactive P incorporated during the subsequent 40-min incubation; this was probably due to a trace of intrinsic kinase activity of the proenzyme. D, densitometric tracing of phosphorylated phosphorylase kinase. The letters α, β, and γ designate the respective subunits of phosphorylase kinase. The arrows α and β represent the origin and dye front of the gel, respectively.

\(^{4}\) Molecular weights of α, β, and γ subunits were estimated to be approximately 14.2 \times 10^3, 13 \times 10^3, and 4.3 \times 10^3, respectively, by the method of Cohen (15). The minimum molecular weight of phosphorylase kinase is calculated to be about 31.5 \times 10^4 and is referred to as a "monomeric unit" of the kinase.

\(^{5}\) After limited proteolysis, the active protein kinase produced showed specific activity of about 1 \times 10^8 units/mg of protein with histone as phosphate acceptor. Under the same conditions, the proenzyme was practically inactive both in the presence and absence of cyclic nucleotides.
O-4, with protein kinase A; and O-0, without protein kinase M activated with either bovine cerebellar protein kinase M (15 units) or the catalytic subunit of bovine cerebellar protein kinase A (20 units) under the conditions described in the text except that protein kinase M is simply a proteolytically modified artifact of cyclic nucleotide-dependent protein kinases.

Therefore, the possibility may be ruled out that protein kinase M showed no tissue specificities. Further studies are under way to elucidate the role of this new protein kinase system in regulating protein phosphorylation reactions.

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